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NUMBER 2

A SUCTION TYPE OF ELECTRODE FOR ELECTRO-ENCEPHALOGRAPHY¹

BY HAROLD V. RICE²

Abstract

A simple and serviceable type of electrode for electroencephalography is described. Its main advantages are the ease and speed with which it can be applied, and its reliability. Its use permits the application of 14 scalp leads to a patient in about 15 minutes or less. The construction and application of the electrode are described.

The type of electrode herein described has been in use in routine electroencephalography for several months and has proved reliable, easy to make, and quick to apply. The first ones made were similar to those described by Andrews (1) but certain features of their design were not satisfactory, notably the fact that they protruded too far above the surface of the skin and tended either to move or be knocked off, and that they were somewhat difficult to apply. The modification that is now used fits closely against the skin so that the patient lies on the electrodes on a pillow without discomfort and without danger of breaking the electrical contact with the skin. The electrodes are also free of movement artifact to a degree comparable with flat electrodes sealed on with collodion.

Each electrode consists of a small piece of lead coated brass tubing, 5 to 8 mm. in diameter and about 5 mm. long, covered at one end with a rubber membrane, and with an enamelled wire soldered to it. One end of the brass tube (which should be thin-walled) is first slightly flanged by hammering with a round-headed hammer or rod of a diameter slightly larger than that of the tube, to simplify the attachment of the rubber diaphragm. The tube is then coated with solder, a length of thin enamelled wire soldered to it, and any exposed copper soldered over. In our experience such lead coated electrodes are stable electrically and do not corrode. When thus prepared, the electrode is completed by tying a piece of rubber over the flanged end. Thick dental dam or even bicycle inner tubing may be used.

In applying the electrode it is necessary to clip the hair from the scalp over an area of about 1 sq. cm. A convenient method is to use a set of barber's clippers from which the teeth at each end have been ground off so that a

¹ Manuscript received September 26, 1944.

Contribution from the Department of Physiology, The University of Manitoba, Winnipeg, Man.

² Assistant Professor.

swath only 1 cm. wide is cut. The electrode is then filled with electrode jelly from a syringe, care being taken to expel all air. The clipped area is rubbed for a moment with electrode paste to soften the skin. The electrode

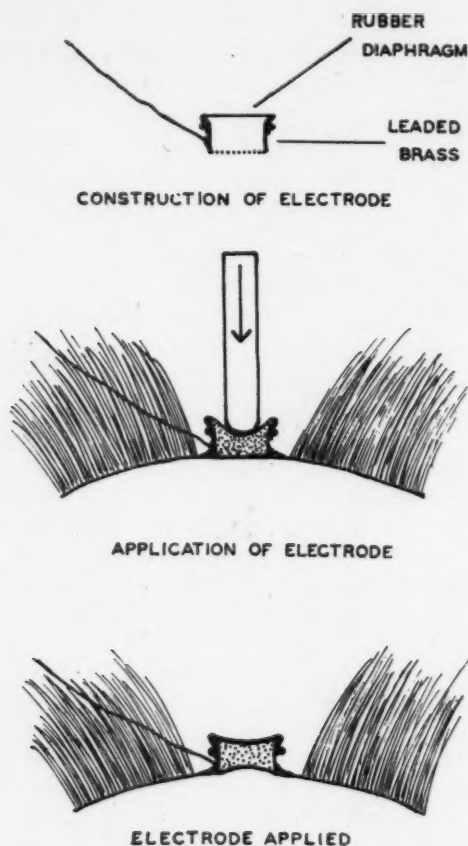


FIG. 1. Construction and application of electrode.

is then applied and the membrane depressed, using a rounded rod. Some electrode paste is thus extruded around the base of the electrode, which is held in place by strong suction when the pressure applied through the rod is removed.

The electrode can be speedily applied. It provides excellent contact since the metal is drawn tightly against the skin. The interelectrode resistance measured through two electrodes is about 10,000 ohms. The efficiency of the contact is readily checked at the end of a test by the amount of force required to remove it, and by the definite mark left when the suction has been

maintained. No sensation of discomfort is caused. Fourteen electrodes can be put on by one person in less than 15 min. so that a routine test with 14 leads rarely takes over an hour.

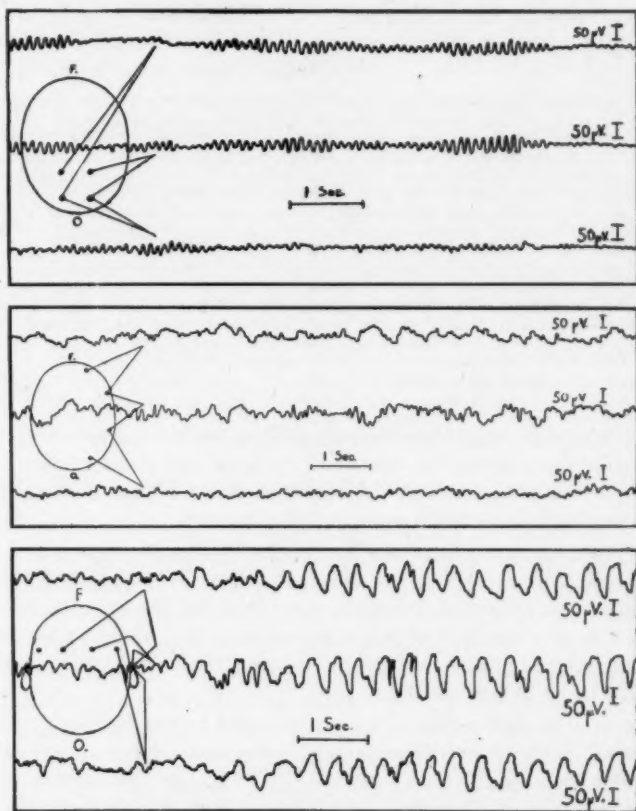


FIG. 2. Sample electroencephalograms taken with the electrodes. Top. Normal bipolar tracings from the occipital region. Centre. Bipolar records from the right side of the head showing phase reversals. A right frontal lobe tumour was found at operation. Bottom. Monopolar records of high voltage three-per-second waves, and waves and spikes brought on during hyperventilation in an epileptic.

The main disadvantage is the need to clip the hair. The small areas are rarely visible, however, and they provide useful landmarks for reference.

Fig. 1 illustrates the construction and application of an electrode. Sample electroencephalograms are shown in Fig. 2.

Reference

1. ANDREWS, H. L. *Am. Heart J.* 17 : 599-601. 1939.

FORMATION AND GROWTH OF BUBBLES IN AQUEOUS SOLUTIONS¹

By J. B. BATEMAN² AND J. LANG³

Abstract

A preliminary study has been made of the evolution of nitrogen and carbon dioxide from various aqueous solutions subjected, in a simple dilatometer, to decreased pressures. The experiments support the view that all aqueous solutions contain a small number of foci for bubble formation. The presence of these foci is shown by a rapid transient evolution of gas immediately following a large rapid reduction of pressure. The nuclei are rather specific in character: the majority of the particles in suspensions of charcoal, blood corpuscles, and various hydrophobic substances, are unable to function in this manner. When decompression occurs in small steps, evolution of gas is mainly attributable to bubbles formed at preferred spots on the vessel wall, and observed variations in rate of growth have been analysed in terms of the nature of the dissolved gases (nitrogen or carbon dioxide), the pressure gradient, and the relative importance of pure diffusion and convection under various conditions. In particular, the observed effect of carbon dioxide in accelerating the growth of nitrogen bubbles has been attributed mainly to its high absorption coefficient rather than to any specific effect upon the number of gas nuclei.

This paper deals with the formation of bubbles in simple and colloidal aqueous solutions containing dissolved nitrogen and carbon dioxide. The approach to the problem and the incompleteness of the study arise from practical considerations which need not be discussed.

In the presence of a gas phase the release of dissolved gases from supersaturated solution is inevitable, although often very slow. Initiation of bubbles in a supersaturated homogeneous liquid, on the other hand, requires the performance of work, and is rather improbable. Thus unless there is a pre-existing gas phase such metastable systems may be of great permanence (see L. Hill (6); Findlay and King (3)).

The metastable condition is usually terminated by the formation of bubbles on the vessel walls or on the surface of some solid object immersed in the liquid (Boyle (6, page 22); see also (5, 12, 13)). In the opinion expressed by Schoenbein (13) in 1837 such interfaces, at first sight remarkably diverse in character, are essentially pre-existing bubbles, since their catalytic effect is abolished when they are freed from adsorbed gas. It appears, however, that most freshly formed surfaces are active, and Fricke attributes their effect to a temporary absence of wetting by the solvent (4, 5).

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Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ont. This work was part of an investigation on decompression sickness carried out under the auspices of the Associate Committee on Aviation Medical Research, National Research Council of Canada.

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Sharp angles, as such, do not act as nuclei. In solutions of carbon dioxide equilibrated at two atmospheres pressure and decompressed to one atmosphere, the quiescent condition is always succeeded, sooner or later, by a "rather rapid spontaneous evolution of gas" (4). It is not clear whether this results from an accidental mechanical disturbance or from the gradual "conditioning" of nuclei by adsorption of dissolved gas.

The suggestion has often been made that the presence of salts and colloids stabilizes supersaturated solutions of gases: Boyle (6, page 22) in 1670 remarked upon the difficulty of producing bubbles by evacuation of blood; Findlay and King (3) observed that dextrin and gelatin greatly prolong the quiescent period preceding evolution of dissolved carbon dioxide, while peptone and ferric hydroxide abolish it. The present experiments, suggested partly by these facts, were designed to throw further light upon the effects of solutes, colloidal dispersed particles, or suspended material, in modifying the process of gas evolution. It was of particular interest to know whether formed elements in blood and blood serum could act as nuclei. Various aqueous systems saturated with nitrogen or nitrogen and carbon dioxide have been examined. The probable number of effective nuclei and its relation to the amount of supersaturation are considered on a semi-empirical basis.

Methods and Procedure

We have determined volumetrically the rate of evolution of gas from solutions previously equilibrated with known partial pressures of nitrogen and carbon dioxide at 37° C. and atmospheric pressure and then subjected to decreased pressures at the same temperature. The change in total volume of the system has been taken as the volume of gas produced.

Saturation at atmospheric pressure was accomplished in a single tonometer of usual type (Austin *et al.* (2)), with great care to avoid contamination with stopcock grease. All glass apparatus was cleaned with chromic acid.

The dilatometer most frequently used consisted of a spherical Pyrex bulb with two openings diametrically situated, one leading to a capillary tube and stopcock through which the liquid could be introduced to the bulb, the other sealed to a graduated, calibrated capillary tube consisting of a portion of small bore, proximal to the bulb, and one of large bore fused to the distal end of the smaller portion. The two limbs were horizontal and co-linear, so that bubbles forming in the bulb would collect at the top without obstructing the capillary openings.

Volume of bulb and connections: 3.13 cm³.

Calibrated tube: graduation 0 to 10 cm. from bulb: average radius, 0.023 cm.; volume 0.018 cm³;

graduation, 10 to 23 cm.: average radius, 0.035 cm.; volume, 0.041 cm³.

The bulb was filled with liquid from the stopcock end, in such a manner as to displace all visible gas, and the open end of the calibrated tube was then connected to a mercury manometer and pump. The thread of liquid extending

into the calibrated tube served for volume measurements; it was found that bubble formation in the capillary thread occurred very seldom, and then only at extremely low pressures. The apparatus was kept at $37 \pm 0.2^\circ \text{C}$. by means of a water bath and thermoregulator.

First Series. Measurements were made successively at about 760, 600, 500, 380, 300, 220, and 120 mm. of mercury, enough observations being made at each pressure to establish accurately the course of gas evolution. At the end, the accuracy of the volume measurements was sometimes checked by taking a Boyle's law curve for recompression of the bubbles. In other cases, the pressure was raised rapidly to atmospheric and the course of gas reabsorption followed.

Second Series. The liquids were suddenly decompressed to 293 mm., volume measurement taken as before, for an appropriate period, and then a further curve was taken at 193 mm.

Third Series. Sudden decompression to 200 mm. Volume measurements were made at the shortest possible intervals. These experiments gave essentially different results from those in the first series.

Results

Series I Reduction of Pressure in Small Stages

Various liquids equilibrated with pure nitrogen, with nitrogen + 12 to 13% carbon dioxide, and with air, were examined.

(1) In most cases a quiescent period after reducing the pressure from 760 to 600 mm. was absent. Evolution of gas was immediate and linear, with occasional exceptions; in one or two cases there was no measurable evolution at 600 mm. (Fig. 1).

(2) Upon further reduction of pressure, there was immediate adjustment to a new constant rate of gas production (Fig. 1). Occasionally there were abrupt changes in rate at constant pressure.

(3) The rate of evolution of gas increased very rapidly with decreasing pressure (Fig. 1).

(4) Gas was evolved more rapidly from solutions equilibrated with nitrogen-carbon-dioxide mixtures than from those containing only nitrogen or air. The difference in rate sometimes became 10-fold at about 150 mm. of mercury.

(5) Bubbles of pure nitrogen formed under reduced pressure were absorbed extremely slowly upon recompression; when carbon dioxide was present, dissolution was very rapid during the first 10 min., leaving presumably a slowly dissolving residue of nitrogen. Solution of the rapidly dissolving portion, of volume V , occurred according to the equation:

$$V = kt^{-0.82}.$$

Absolute rates of gas formation were difficult to reproduce, even when special care was taken in cleaning the apparatus. The variations were greatest for pure water; the rates showed a five-fold range, for example, in

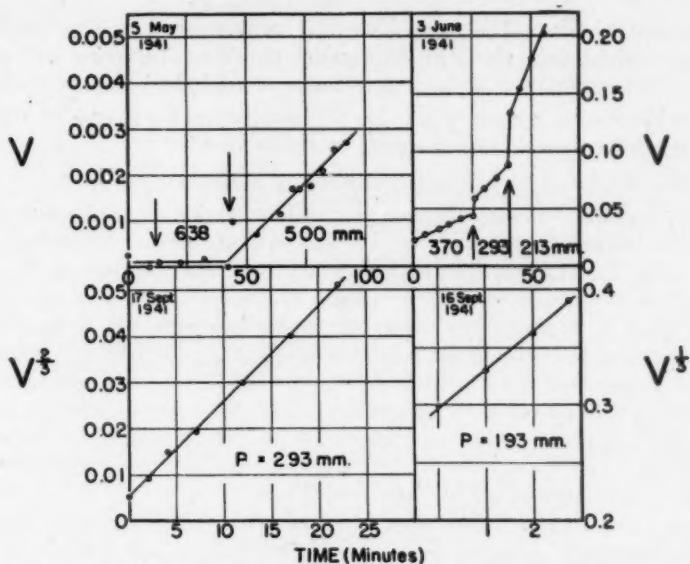


FIG. 1. Evolution of dissolved gas from solutions at 37° C. under reduced pressure.

- 5 May: Water initially in equilibrium with air at atmospheric pressure and 37° C. Abscissa: time, in minutes. Ordinate: volume of gas, in cm.³ Pressure reduced in steps. Illustrates period of no bubble formation at 638 mm. followed by immediate linear evolution of gas when pressure reduced to 500 mm. of mercury.
- 3 June: Suspension of finely ground Kahlbaum "Carbo activ granulat" in distilled water. Initially in equilibrium with air at atmospheric pressure and 37° C. Abscissa: time, in minutes. Ordinate: volume of gas, in cm.³ Pressure reduced in steps. Illustrates linear evolution of gas and rapid increase in rate with reduction of pressure.
- 17 Sept.: Water initially in equilibrium with nitrogen and 13% of carbon dioxide at atmospheric pressure and 37° C. Ordinate: (volume of gas)^{1/2}. Sudden reduction of pressure to 293 mm. of mercury.
- 16 Sept.: Same solution as 17 Sept. Ordinate: (volume of gas)^{1/2}. Reduction of pressure to 193 mm. of mercury after a period of gas evolution at 293 mm. of mercury.

nitrogen evolution at the lower pressures, and a somewhat greater range in presence of carbon dioxide. Apparently most of this variation lay in the state of the vessel wall, since points of origin of bubbles were often visible. Very careful cleaning considerably reduced such variations, while the presence of a wetting agent in the water (such as 0.5% "Aerosol O.T.") gave rise to a much more uniform low rate of gas formation, particularly in presence of carbon dioxide. Emulsions of non-polar substances stabilized by Aerosol liberated gas at about the same rate as aqueous solutions of Aerosol; this was shown for emulsions of kerosene and ethyl palmitate, and for petrolatum emulsified with Aerosol and cetyl alcohol.

Blood and blood plasma appear to act in much the same way as Aerosol, when allowance is made for differences in viscosity, while dialyzed colloidal ferric hydroxide and aqueous suspensions of Kahlbaum bone charcoal or ivory black are scarcely distinguishable from pure water. On the other hand,

visible matter in suspension often materially increases the rate of gas evolution; this was noticed particularly by determining the effect of filtering such matter from a dilute solution of sodium bicarbonate of analytical reagent grade.

Thus the results appear to provide no justification for the belief that colloidal particles are able to any extent to act as nuclei.

Series 2 Measurements at 293 and 193 mm. of Mercury

Water equilibrated with nitrogen plus carbon dioxide: rate of gas evolution gradually increased with time, t . In most cases, a plot of $V^{\frac{1}{2}}$ against t was linear (Fig. 1). In two instances in which this was the case at 293 mm., the data at 193 mm. gave a linear relation between $V^{\frac{1}{2}}$ and t (Fig. 1). Whenever a $V^{\frac{1}{2}}$ or $V^{\frac{1}{3}}$ linearity was observed, the bubbles were seen to be adhering to various points of the vessel wall throughout the experiment. Whenever, through mechanical shock or other means, the bubbles were caused to become detached and to collect at the top of the vessel, the curves tended to become linear in V , as in the earlier experiments, or to show a progressive flattening. The absolute rates of change of V were greatly variable and dependent upon the previous treatment of the containing vessel.

Blood plasma equilibrated with nitrogen plus carbon dioxide: here the bubbles invariably collected at the top of the vessel, and the curves of V against t were either linear or concave toward the t -axis (exponent of V greater than 1.0).

Ivory black suspended in water, equilibrated with nitrogen plus carbon dioxide: very high initial rate of volume change, causing effervescence, attaining constancy after one to three minutes. This observation suggested that nuclei present in the liquid phase were being carried to the surface and thus rendered ineffective in further bubble formation; it seemed likely that the same process might occur to a smaller extent in other liquids. Accordingly the third series of experiments was made, in which the time course of gas evolution during the first minute was followed in detail.

Series 3 Sudden Decompression to 200 mm. of Mercury

The transient effect of nuclei is clearly shown in Fig. 2. The effect is quite significant for distilled water, considerably more so for blood, and most marked for ivory black. The effect was not diminished by using plasma instead of whole blood, nor increased by using packed corpuscles; the nuclei are apparently not sedimentable. Gas-free water shows a small volume change, 0.001 cm³., 10 times that expected from the compressibility of water and presumably referable to deformation of the glass bulb. Perhaps the greater duration of the transient effect in a suspension of ivory black is due to a distribution of particle size, while the suddenness of the effect in water and blood suggests that only a limited number of almost identical particles is involved. The average time of transit of a small bubble to the top of the bulb is probably of the order of 0.1 sec. (Luchsinger (9)), so that the time required for a new bubble to form at a nucleus can vary between a fraction of a second and one minute in the case of the charcoal suspensions.

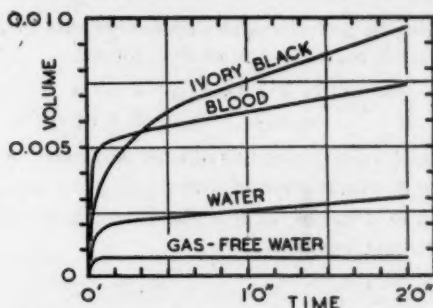


FIG. 2. Time course of evolution of gas from various liquids equilibrated with a mixture of nitrogen and carbon dioxide at atmospheric pressure and suddenly decompressed to 200 mm. of mercury. Abscissa: time, in minutes. Ordinate: gas volume, in cm.³.

Discussion

We wish, in discussing the above data, to decide to what extent the observed rapid increase in rate of gas evolution with decreasing pressure is either implicit in the laws of diffusion into a small constant number of bubbles or requires a rapid increase in the number of effective nuclei.

The rate of growth of a bubble of radius a by diffusion of a single dissolved gas from the surrounding liquid is given by:

$$r = dV/dt = 4\pi a \cdot \alpha_1 \delta_1 \cdot T(p'_1 - p_1)/273p \quad (1)^*$$

where V = Volume under conditions of measurement.

t = Time.

α_1 = Absorption coefficient = 0.0127 for nitrogen in water at 37° C. (Van Slyke, Dillon, and Margaria (14)).

δ_1 = Diffusion coefficient of dissolved gas = $1.4 \cdot 10^{-3}$ cm.² per min. for nitrogen in water, extrapolated to 37° C. from data of Hufner (7).

p'_1 = Gas pressure which would be in equilibrium with dissolved gas.

p_1 = Partial pressure of gas in bubble.

p = Total pressure in bubble; this may be assumed equal to the hydrostatic pressure around a bubble large enough to satisfy the condition: $p \gg 2\sigma/a$, where σ is the interfacial tension.

For N bubbles of equal size,

$$a = (3V/4\pi N)^{1/3}$$

so that Equation (1) becomes

$$r = dV/dt = 6.135N^{1/3}V^{1/3}\alpha_1\delta_1(p'_1 - p_1)/p, \quad (2)$$

where the numerical term 6.135 includes a factor $\ln 2$, or 0.693, as a correction for portions of the bubble surface in contact with the vessel wall.

*For derivation compare Mache (10).

Since we are dealing in practice with diffusion of nitrogen, carbon dioxide, and water, this equation must be extended as follows:

$$r = r_1 + r_2 + r_3 = 6.135 N^{\frac{1}{2}} V^{\frac{1}{2}} [\alpha_1 \delta_1 (p_1' - p_1) + \alpha_2 \delta_2 (p_2' - p_2) + K.f(V) (p_3' - p_3)] / p, \quad (3)$$

where the suffixes 2 and 3 refer to carbon dioxide and water, K being a constant representing the rate of evaporation of water.

For steady growth of a bubble over a short period, the proportions of the components must remain constant:

$$r_2 = r_1 p_2 / p_1 \quad r_3 = r_1 p_3 / p_1,$$

so that

$$r = r_1 \cdot [1 + (p_2 + p_3) / p_1] = r_1 p / p_1, \quad (4)$$

since

$$p = p_1 + p_2 + p_3.$$

Approximately, we may consider p_3 nearly equal to p_3' , because the rate of evaporation of water is certainly very large compared to the rate of diffusion of dissolved gases, and p_2 not greatly different from p_2' , because of the high absorption coefficient, α_2 , of carbon dioxide. For most of our experiments p_2' was either zero or roughly 100 mm. of mercury; p_3' was always about 50 mm. of mercury. Substituting these numerical values and combining Equations (3) and (4), we find

$$r = dV/dt = 1.09 \cdot 10^{-4} V^{\frac{1}{2}} N^{\frac{1}{2}} [p_1' - (p - 50)] / (p - 50)$$

or

$$d(V)^{\frac{3}{2}}/dt = 7.28 \cdot 10^{-5} \cdot N^{\frac{1}{2}} [p_1' - (p - 50)] / (p - 50) \quad (5a)$$

for nitrogen bubbles, and

$$d(V)^{\frac{3}{2}}/dt = 7.28 \cdot 10^{-5} \cdot N^{\frac{1}{2}} [p_1' - (p - 150)] / (p - 150) \quad (5b)$$

for nitrogen-carbon-dioxide bubbles.

This gives the linearity in $V^{\frac{1}{2}}$ observed in the second series of experiments at 293 mm. of mercury. Calculation of N , using Equations (5), gives values around 10 for water in a well cleaned container; when the walls were very dirty, in one case, N rose to nearly 500.

Linearity in $V^{\frac{1}{2}}$, observed at 193 mm., can be explained by convection associated with the rapid growth of bubbles and the evaporation of water. Transfer of gas in a stirred liquid is determined less by δ than by k , the transfer coefficient per square centimetre of interface:

$$dV/dt = Nk \cdot 4\pi a^2 (p_1' - p_1) / p, \quad (6)$$

giving

$$d(V)^{\frac{3}{2}}/dt = 1.61 N^{\frac{1}{2}} k (p_1' - p_1) / p. \quad (7)$$

This cannot be used, however, in calculating N since k is subject to enormous variations; using Adeney and Becker's (1) value, for a bubble in violently stirred water, we obtain a ridiculously small value of N .

In discussing the variation of N with total pressure, it is necessary to modify Equation (3) empirically to account for the fact that r was nearly always

independent of V in the experiments of Series I. We can do this by writing:

$$r = \frac{1}{p} \cdot f(N)[\alpha_1 \delta_1 (p_1' - p_1) + \dots], \quad (8)$$

which gives $f(N) = br$, (9)

where $b = (p - 150)/\alpha_1 \delta_1 [p_1' - (p - 150)]$ (9a)

for bubbles containing nitrogen, carbon dioxide, and water vapour,
and $b = (p - 50)/\alpha_1 \delta_1 [p_1' - (p - 50)]$ (9b)

for bubbles containing only nitrogen and water vapour.

In these cases, p_1' is calculated from the original nitrogen pressure with which the liquid was equilibrated, $(p_1')_0$, as follows:

$$p_1' = (p_1')_0 - 273 p_1 V / 310 \alpha_1 v, \quad (10)$$

where V = volume of gas, v = volume of liquid = 3 cm³.

Values of $f(N)$ have been calculated for all experiments in which r was constant. Certain of these results are given in Table I, in order to illustrate the following points:

(1) $f(N)$ rises with decreasing pressure; there may be a five- to seven-fold increase between 600 and 200 mm. of mercury. This rise is in our opinion

TABLE I

EVOLUTION OF GAS FROM VARIOUS SOLUTIONS EQUILIBRATED WITH NITROGEN OR NITROGEN WITH 13% CARBON DIOXIDE

Solution	Nitrogen				Nitrogen with 13% carbon dioxide			
	p	$10^3 r$	$n \cdot f(N)$	Equation	p	$10^3 r$	$n \cdot f(N)$	Equation
Emulsion of kerosene in 0.5% Aerosol	597	0.6	1.2	9b	597	0.3	0.5	9a
	493	2.3	2.3		501	1.6	1.3	
	383	4.4	2.4		383	8.3	3.1	
	300	12.3	4.3		300	20.0	4.0	
	221	32.0	7.1		223	77.0	6.2	
					165	460.0	~7.0	
0.01 M NaHCO ₃	598	0.5	1.0	9b	588	0	0	9a
	495	2.5	2.6		492	0.4	0.3	
	396	4.5	2.6		392	2.9	1.1	
	298	11.3	3.8		289	12.9	2.3	
	217	29.3	6.5		206	56.0	3.4	
	137	85.0	9.3		123	2000	(72)	
								11
Blood	592	0.5	3.0	9b	597	0.6	2.9	9a
	499	0.6	2.0		495	1.7	4.2	
	377	3.3	5.1		375	3.4	3.6	
	295	5.7	6.0		299	8.3	5.1	
	219	13.5	9.0		219	39.0	8.1	
	148	43.0	15.6		141	200	(72)	
								11

$r = dV/dt$ in cm.³ per min. for spherical vessel containing 3 cm.³ of liquid.

$f(N)$ defined in text.

n = viscosity = 3 for blood and 1 in other cases.

sufficiently accounted for by the collection of bubbles at the top of the vessel, and in part also by the increasing importance of water vapour, which can enter over the entire surface of all the bubbles present, while entry of carbon dioxide and nitrogen is confined to those parts of the surface in contact with considerable thicknesses of liquid.

(2) Despite the greater absolute values of r when carbon dioxide is present, $f(N)$ is usually somewhat smaller. Thus carbon dioxide certainly does not increase the number of nuclei under the conditions of these experiments.

A further case requires mention:

when $p_2' + p_3' > p$

the bubbles continue to grow by diffusion of carbon dioxide and water, so that the partial pressure of nitrogen is no longer a controlling factor in the rate of growth.

In this case

$$r = f(N)\alpha_2\delta_2(p_2' - p_2)(1 + p_3/p_2)/p, \quad (11)$$

where $\alpha_2 = 0.545$ (Van Slyke, Sendroy, Hastings, and Neill (15))

$\delta_2 = 1.4 \cdot 10^{-3}$ cm² per min. extrapolated from Hüfner's values (7).

Equation (11) cannot be applied with accuracy, since we do not know p_1 , and hence cannot calculate p_2 , but rough calculations give rather high values of $f(N)$ (Table I); these are probably due to the streaming set up by expansion of the bubbles during reduction of pressure.

To recapitulate: probably all aqueous solutions contain a certain relatively small number of particles able to act as nuclei. In a stationary liquid bounded by a container and subjected to gradually decreasing pressure, these nuclei are gradually carried to the top of the vessel by the bubbles originating on them; this process occurs so slowly that the rate of gas evolution is determined mainly by the rate of growth of bubbles which originate on the wall of the vessel, since these can grow to a considerable size before rising to the surface. The suspended nuclei can be made evident only by a sudden large reduction of pressure; under these circumstances, the nuclei all become effective within a short time, and are thus able to produce a very rapid temporary evolution of gas. Aside from the transient effect of these nuclei, the observed variation of rate of gas evolution with pressure can be roughly accounted for theoretically, with the aid of a diffusion equation, merely by assuming the number of bubbles to increase gradually during the experiment, as a result of detachment from their nuclei on the vessel wall and failure to coalesce at the top of the vessel. At no stage is there such a rapid increase in rate of gas evolution as to suggest that widespread cavitation of the homogeneous liquid occurs; nor indeed, would such a result appear very probable on theoretical grounds (see Landau and Lifshitz (8); but compare Piccard (11)). Concerning the nature of the suspended nuclei, it is clear that they must be of rather specific character. The numerous suspended elements in blood do

not act as nuclei to any extent, nor again do the majority of the particles in a suspension of ivory black, although an increased initial rate of gas formation is certainly associated with the presence of the latter particles. The nuclei are also not removed from blood or plasma by centrifuging. In the single case of a solution of sodium bicarbonate, which had shown an inordinately high rate of gas formation at the lowest pressures, the nuclei appeared to be largely removed by filtration.

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RESPIRATORY QUOTIENTS OF EXPIRED AND ALVEOLAR AIR AT NORMAL AND REDUCED BAROMETRIC PRESSURES¹

By J. K. W. FERGUSON² AND L. P. DUGAL³

Abstract

In the paper are reported the results of 55 experiments on four subjects seated quietly at ground level and 15,000 ft. breathing air. Inspiratory and expiratory alveolar air samples were taken immediately before and after a period during which expired air was collected.

A systematic difference can be demonstrated between the respiratory quotients of inspiratory and expiratory samples of alveolar air taken when the subject is seated at rest. This difference is much diminished by anoxia at a barometric pressure equivalent to 15,000 ft. supporting Krogh and Lindhard's analysis of the course of gaseous exchanges in the lungs. The $R.Q.$'s of expiratory samples of alveolar air approximate closely those of expired air. Haldane's hypothesis that the respiratory dead space for carbon dioxide is smaller than for oxygen is not supported by our findings under seated resting conditions on the average. The $R.Q.$ of expired air may be used for calculating average relations between the partial pressures of carbon dioxide and oxygen in alveolar air by the Alveolar Equation.

Introduction

Interest in the respiratory quotient ($R.Q.$) of alveolar air is due mostly, at the present time, to its occurrence in the Alveolar Equation, which is, in fact, a rearrangement of the formula long in use for calculating $R.Q.$'s of both expired and alveolar air. The equation has been used extensively to predict and describe the physiological conditions produced at different altitudes by breathing different mixtures of oxygen and nitrogen (2, 9). That the Alveolar Equation is valid enough to be very useful has been demonstrated fairly satisfactorily by analyses of alveolar air and arterial blood over a wide range of altitudes (9). To assess its validity, however, as an accurate description of physiological relations it is necessary to consider the discrepancies that have been reported between the $R.Q.$'s of alveolar air and those of expired air (6, 7, 10). These must be considered too in the light of discrepancies said to occur between $R.Q.$'s of alveolar air taken at different times in the respiratory cycle (12).

The $R.Q.$ is the volume of carbon dioxide added to a given volume of inspired air, divided by the volume of oxygen removed from it in the process of respiration. If expired air is a mixture of alveolar air and unmodified inspired air—namely the last part of the inspired air that remains in the so-called 'dead space', then the $R.Q.$ of alveolar air and of expired air must be identical. Haldane, however, found that the alveolar $R.Q.$ was consistently lower than

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the $R.Q.$ of expired air in the one subject on which he presented data (7). Similar results were apparently not unusual in the experiments of Krogh and Lindhard (12, 14), who inferred that the method of sampling was responsible for the difference, while Haldane concluded that the results indicated that the respiratory dead space was smaller for carbon dioxide than for oxygen.

Haldane's argument is long and difficult to follow as it differs substantially in three different publications (6, 7, 8). It is based partly on the anatomical arrangement of the respiratory bronchioles and atria and partly on the fact that carbon dioxide diffuses more readily through tissues than does oxygen and hence might diffuse into parts of the respiratory dead space from which the uptake of oxygen would be much slower. For brevity Haldane's concept will be referred to in this paper as the *Spatial Effect* in so far as it affects the agreement between the $R.Q.$'s of expired and alveolar air. To avoid digression into a complicated subject it need be stated here only that equality of $R.Q.$'s of expired and alveolar air implies equality of dead space as calculated from carbon dioxide and from oxygen.

Krogh and Lindhard (13) present evidence that the rate of transfer of oxygen and carbon dioxide between the blood and the alveolar air is not uniform throughout the respiratory cycle. Towards the end of the cycle (i.e. after the end of expiration) the rate of elimination of carbon dioxide is retarded as compared with the rate of oxygen absorption. This would result in lower $R.Q.$'s in samples taken at the end of expiration. According to this view agreement or disagreement between the $R.Q.$'s of expired and alveolar airs would depend on the time of sampling the alveolar air. The phrase, *Time Effect*, will be used in the rest of this paper in reference to the concept of Krogh and Lindhard.

It is now apparent that before any assessment can be made of the *Spatial Effect* on $R.Q.$'s, the *Time Effect* must be eliminated in some way. Theoretically this might be done by timing the sampling of the alveolar air so that only *mean* alveolar air was collected. It is hard to see how this could be done. However, it occurred to us that the *Time Effect* might be diminished or abolished under conditions of anoxia. Such conditions should not interfere with the *Spatial Effect*.

The reason for expecting the *Time Effect* (if demonstrable) to disappear during anoxia follows from the argument of Krogh and Lindhard who attribute the relative retardation of the output of carbon dioxide at the end of expiration to approaching equilibrium between the carbon dioxide in the blood and in the alveolar air. At this time, at ground level, the partial pressure of oxygen in the alveoli is still appreciably higher than that of arterial blood so that absorption of oxygen continues. Undoubtedly this is an oversimplified statement of the case but if it is even partly correct, anoxia should alter the circumstances considerably since it has been shown (3, 4, 5) that during anoxia oxygen behaves more like carbon dioxide in that the partial pressure of oxygen, in the alveoli at the end of expiration, approaches very closely that in arterial blood.

Methods

Two samples of alveolar air were collected, by the Haldane-Priestly technique, one at the end of expiration and another at the end of inspiration. Expired air was then collected in a Douglas bag for several minutes after which two more samples of alveolar air were taken.

Five subjects were used. One was a physiologist, one a technical assistant, and the other three flying personnel in training. Considerable time was devoted to training each subject in the method of giving alveolar air samples and in every experiment pneumograph tracings were taken to ensure as far as possible that breathing was normal and the timing of the sample correct (see Fig. 1.). One subject had difficulty in learning the technique and never did give satisfactory respiratory tracings throughout an experiment. His $R.Q.$'s also indicated that he consistently over-ventilated during the sampling technique. The results on this subject were not included in the averages.

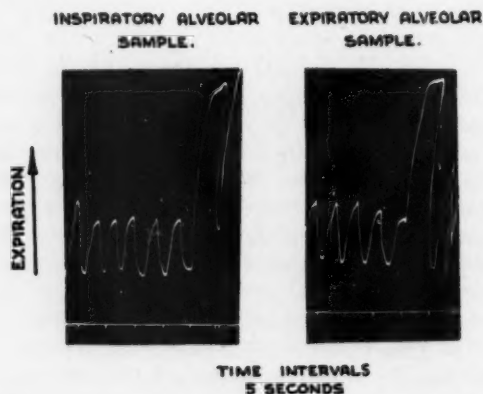


FIG. 1. *Pneumograph tracings during collection of alveolar air samples.*

Since ventilation of a decompression chamber is never perfect, the subjects breathed air from an oxygen demand regulator (R.C.A.F. Type C3B) connected to a cylinder of compressed air. The regulator supplied the air only during inhalation, at pressures within 1 mm. Hg of the ambient pressure in the chamber (see Fig. 2). The composition of the compressed air was checked by frequent analyses.

The subject wore a Type C2 R.C.A.F. oxygen mask while inspiring the compressed air from the demand regulator. The mask was removed with one hand while the alveolar air sample was delivered through a Heidbrink sampler into a mercury sampling bottle (Brodie type). The mask was replaced before the next breath was inhaled.

The order of a typical experiment is summarized as follows and was identical at both ground level and 15,000 ft.

- (1) Chamber air was breathed at prevailing pressure for 20 min.
- (2) Air from the cylinder was breathed for 15 min. at prevailing pressure as delivered from the demand regulator, while the subject was wearing the mask.

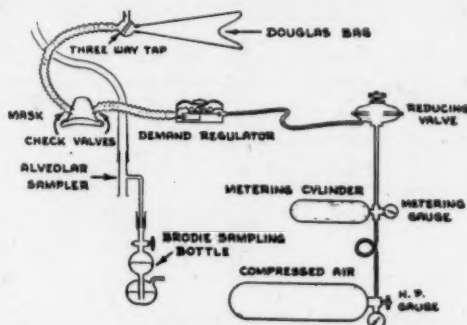


FIG. 2. System for studying inspired, expired, and alveolar air.

- (3) An alveolar air sample was taken at the end of expiration followed in 1 to 1½ min. by an alveolar air sample at the end of inspiration.
- (4) Expired air was collected for 4 to 8 min.
- (5) Another alveolar air sample was taken at the end of expiration followed in 1 to 1½ min. by another at the end of inspiration.

After thorough mixing in the Douglas bag, two samples of expired air were drawn into mercury samplers (Brodie type) for analysis in Haldane analysers with fully calibrated, 10 cc. burettes.

After a number of experiments it became obvious that our results at ground level were not agreeing with those of Haldane in finding that the alveolar *R.Q.* was consistently lower than the *R.Q.* of expired air. We wondered whether taking expiratory alveolar samples before the inspiratory sample particularly at the short interval of 1 to 1½ min. might be affecting the result. The order of taking the inspiratory and expiratory alveolar samples was reversed in 20 experiments without any effect on the trend of the results. All experiments are consequently combined in the averages.

It will be noted that at 15,000 ft. the subject was exposed to anoxia for 35 min. before any respiratory measurements were made. By this time according to Lutz and Schneider (15) the alveolar *R.Q.* should be fairly stable.

Observations

In Table I are shown the *R.Q.*'s of inspiratory and expiratory samples of alveolar air taken at ground level and 15,000 ft. For all subjects at ground level the inspiratory alveolar *R.Q.* is greater than the expiratory alveolar *R.Q.* and the average difference for the series is highly significant. At 15,000 ft. the average difference is much smaller but still significant. Another fact of interest is shown by the standard deviation of differences at the two altitudes.

TABLE I

ALVEOLAR RESPIRATORY QUOTIENTS

Comparison of samples taken at end of inspiration (Insp.) and end of expiration (Exp.)

Subject	<i>F_a</i>	<i>F_e</i>	<i>T</i>	<i>P</i>	Weighted averages
<i>Ground level</i>					
No. of experiments	8	7	8	6	
Av. Insp. <i>R.Q.</i>	.865	.865	.907	.876	.879
Av. Exp. <i>R.Q.</i>	.817	.842	.889	.829	.845
Difference	.048	.023	.018	.047	.034

Standard deviation of differences = .063

Standard error of mean difference = .0115

t = 2.96*p* = less than .01.*15,000 ft.*

No. of experiments	6	7	9	4	
Av. Insp. <i>R.Q.</i>	.876	.877	.905	.858	.884
Av. Exp. <i>R.Q.</i>	.877	.867	.883	.840	.871
Difference	-.001	.010	.022	.018	.013

Standard deviation of differences = .033

Standard error of mean difference = .0064

t = 2.03*p* = .05

It is apparent that the variability of individual experiments is much less at 15,000 ft. than at ground level.

Table II shows inspiratory alveolar *R.Q.*'s compared with *R.Q.*'s of expired air. At ground level the alveolar *R.Q.*'s tend to be higher than the *R.Q.*'s of expired air, but the tendency is hardly significant on account of the variability of individual experiments. At 15,000 ft. the average difference is smaller and again hardly significant although the variability of individual experiments is much smaller. Only one subject, *F_a*, shows at 15,000 ft. the trend (found consistently by Haldane) for the Alveolar *R.Q.* to be lower, and even on this one subject the mean difference is not significant.

Table III shows expiratory alveolar *R.Q.*'s compared with *R.Q.*'s of expired air. This time the average differences are very small and definitely not significant at either altitude.

TABLE II
INSPIRATORY ALVEOLAR R.Q.'s AND R.Q.'s OF EXPIRED AIR

Subjects	F_a	F_e	T	P	Weighted averages
<i>Ground level</i>					
No. of experiments	8	7	8	6	
Av. Insp. R.Q.	.865	.865	.907	.876	.879
Av. R.Q. of exp. air	.830	.854	.860	.847	.848
Difference	.035	.011	.047	.029	.031
Standard deviation of differences = .088 Standard error of mean difference = .0164 $t = 1.89$ $p = \text{between .1 and .05}$					

15,000 ft.

No. of experiments	6	7	9	4	
Av. Insp. R.Q.	.876	.877	.905	.858	.884
Av. R.Q. of exp. air	.904	.856	.859	.836	.865
Difference	-.028	.021	.046	.028	.019
Standard deviation of differences = .05 Standard error of mean difference = .01 $t = 1.9$ $p = \text{between .1 and .05}$					

TABLE III
EXPIRATORY ALVEOLAR R.Q.'s AND R.Q.'s OF EXPIRED AIR

Subjects	F_a	F_e	T	P	Weighted averages
<i>Ground level</i>					
No. of experiments	8	7	8	6	
Av. Exp. R.Q.	.817	.842	.889	.829	.845
Av. R.Q. of exp. air	.830	.854	.860	.847	.848
Difference	-.013	-.012	.029	-.018	-.003
Standard deviation of differences = .07 Standard error of mean difference = .013 $t = 0.23$ $p = \text{greater than .8}$					
15,000 ft.					
No. of experiments	6	7	9	4	
Av. Exp. R.Q.	.877	.867	.883	.840	.871
Av. R.Q. of exp. air	.904	.856	.859	.836	.865
Difference	-.027	.011	.024	.004	.006
Standard deviation of differences = .042 Standard error of mean difference = .0083 $t = 0.722$ $p = \text{between .4 and .5}$					

For completeness Table IV is added, showing average alveolar *R.Q.*'s compared with expired *R.Q.*'s. As might be deduced from the two preceding tables, the differences are small and not significant. There is some suggestion that different subjects might have shown a systematic difference if more experiments had been done. However, in only one subject in one series was the individual trend consistent enough to be significant (Table II, Subject *T*, ground level). This subject, incidentally, shows an opposite trend to that found by Haldane.

TABLE IV

AVERAGE OF INSPIRATORY AND EXPIRATORY ALVEOLAR *R.Q.*'S AND *R.Q.*'S OF EXPIRED AIR

Subjects	<i>F_a</i>	<i>F_e</i>	<i>T</i>	<i>P</i>	Weighted averages
<i>Ground level</i>					
No. of experiments	8	7	8	6	
Av. alveolar <i>R.Q.</i>	.841	.854	.898	.852	.862
<i>R.Q.</i> of exp. air	.830	.854	.860	.847	.848
Difference	.011	.000	.038	.005	.014

Standard deviation of differences = .085

Standard error of mean difference = .016

t = 0.875 *p* = .4*15,000 ft.*

No. of experiments	6	7	9	4	
Av. alveolar <i>R.Q.</i>	.876	.872	.894	.849	.877
Av. <i>R.Q.</i> of exp. air	.904	.856	.859	.836	.865
Difference	-.028	.016	.035	.013	.012

Standard deviation of differences = .047

Standard error of mean difference = .0093

t = 1.3 *p* = .2

Discussion

Our results support the view of Krogh and Lindhard that the *R.Q.* of alveolar air varies with time in the respiratory cycle. Our finding that anoxia diminishes the Time Effect also supports their explanation of it as paraphrased in the introduction. Other considerations, however, make one very hesitant to accept the explanation as it stands. For the time being, it seems wiser to stress the fact rather than its explanation.

With the Time Effect reduced to negligible proportions by anoxia, there remains no evidence of the Spatial Effect postulated by Haldane. Since it is hard to see how anoxia could diminish the hypothetical Spatial Effect, there seems to be no reason now to postulate that the dead space for carbon dioxide is significantly smaller than for oxygen.

Apart from accidental variations attributable to experimental error, the $R.Q.$'s of *expiratory* samples of alveolar air appear to coincide very closely with those of expired air in the resting state. This may not be the case during muscular work which is a matter for further investigation. For the present we may be satisfied that when the respiratory dead space of a subject is known at rest and it is inconvenient to collect alveolar air—for example, during severe anoxia—expired air could be used with considerable confidence to estimate the alveolar gas tensions, particularly of expiratory alveolar air samples. This is all the more satisfactory since Dill *et al.* (3, 4, 5) have shown that the gas tensions of expiratory samples agree very closely with tensions in arterial blood, particularly during anoxia.

No consideration was given in the introduction to the question of unequal ventilation of different groups of alveoli. Inequalities of alveolar ventilation, as postulated by Haldane (8) and supported very convincingly by the experiments of Roelsen (16) and of Sonne (17), can hardly play much part in the Time Effect as defined in this paper, since we have compared alveolar samples taken at different times but after maximum expiration following similar inspirations. If it is contended that local inequalities of ventilation might in some way be responsible for the different findings at 15,000 ft., i.e., the diminution in Time Effect, it may be countered that at 15,000 ft. the breathing is somewhat deeper and, according to Sonne's findings, deeper inspirations accentuate the inequalities.

Since the criterion of marked inequality of alveolar ventilation is accentuated lowering of oxygen content and $R.Q.$ in the later fractions of a single maximum exhalation (16), inequality of alveolar ventilation may well be responsible in some persons for the $R.Q.$'s that are lower in alveolar air than in expired air, which were reported by Haldane and demonstrated consistently in his one subject. Perhaps all of our subjects had lungs that were too healthy to show such a tendency.

Rather large accidental errors are to be expected in the determination of an $R.Q.$ since it is a quotient of two differences, of four measurements each subject to some error. In addition, it is evident that accurate timing of the sampling of alveolar air is essential for obtaining consistent results, particularly at ground level. The tendency in a subject to involuntary alteration of breathing just before alveolar samples are given is one that is hard to control, and must contribute considerably to irregularity of results. More consistent results could probably have been obtained with subjects lying in a relaxed position rather than sitting up. The erect posture has been shown to influence the alveolar air, tending to lower the tension of carbon dioxide by a kind of relative hyperventilation (11). The effect of posture on alveolar $R.Q.$'s has not yet been determined. Variations of interest might well be found. In the meantime, our conclusions can only be applied to results obtained when the subject is in the seated posture and carrying on minimal activity. But in doing so, it is well to keep in mind that the work on comparison of alveolar

gas tensions and tensions in arterial blood appears to have been done for the most part on subjects that are in the recumbent position (3).

A report on the *R.Q.*'s of alveolar and expired air (1) was received from The Mayo Medical Unit after our experiments had been completed. It was gratifying to find the general conclusions of the two reports so concordant. Among the four subjects used at the Mayo Unit, one showed alveolar *R.Q.*'s consistently lower than those of expired air, one showed a tendency for the alveolar *R.Q.*'s to be higher while the other two subjects showed the alveolar and expired air *R.Q.*'s to agree on the average.

Conclusions

A systematic difference can be demonstrated between the *R.Q.*'s of inspiratory and expiratory samples of alveolar air taken when the subject is seated at rest.

This difference is much diminished by anoxia at a barometric pressure equivalent to 15,000 ft., supporting Krogh and Lindhard's analysis of gaseous exchanges in the lungs.

The *R.Q.*'s of expiratory samples of alveolar air approximate closely those of expired air on the average.

Haldane's hypothesis that the respiratory dead space for carbon dioxide is smaller than for oxygen is not supported by our findings under seated resting conditions.

The *R.Q.* of expired air may be used for calculating average relations between the partial pressures of carbon dioxide and oxygen in alveolar air by the Alveolar Equation.

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DETERMINATION OF BROMIDE-BROMINE IN THE BLOOD OF PATIENTS UNDERGOING TREATMENT WITH BROMIDE¹

BY A. T. CAMERON² AND JEAN S. GUTHRIE³

Abstract

A method is described for the estimation of bromide-bromine in blood, which depends on liberation of bromine by persulphate in sulphuric acid from a zinc hydroxide filtrate from blood, aspiration of the bromine into iodide, and titration of the liberated iodine with thiosulphate. The method is not applicable to normal blood, but is specially designed for estimation of the blood bromine of patients to whom bromides are being administered. It is not suitable for estimation of corresponding amounts of iodide-iodine.

Introduction

Many methods have been published for estimation of bromine in blood and tissues. Usually organic matter is initially oxidized. Many of the procedures give inaccurate results, as Neufeld (7), for example, has pointed out.

It is frequently necessary to measure the blood bromide of mental patients and others to whom bromide has been administered, as when actual bromide intoxication is suspected, or when there seems a possibility that further administration of bromide may produce it. For this type of case there is no need of incineration or similar procedures, since bromide intoxication depends upon the presence of bromide ions as such, while, in any case, the amount of organic bromine compounds formed in blood and tissues after administration of bromide is almost certainly negligible.

To be suitable for the purpose stated, a method should be rapid, easily performed, and moderately accurate for amounts of bromine of the order 50 to 300 mgm. per 100 cc. blood. It need not estimate amounts of the order normally present in blood (about 1 mgm. per 100 cc.).

A method that has been frequently used with mental patients is based upon the development of a brown to brown-red colour when gold chloride is added to a protein-free filtrate of serum or plasma. It was originally described by Walter in 1925 (13, 14) and subsequently modified by Wuth (16), Diethelm (1), Tod (12), and others. Gray and Moore (5) have critically examined it. They find that, as it is usually employed, the average difference between two determinations on the same serum is 20%, while differences as great as 37% may occur. When a photoelectric colorimeter is used, duplicate determinations are usually in good agreement. However, the development of the colour is not specific for bromide; other chemically unrelated substances can

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give a positive reaction resulting in inaccurate and too high values. Gray and Moore consider that the clinical condition of the patient is of more value as a diagnostic and therapeutic index than isolated determinations of blood bromide by the Walter method (Cf. also Fremont-Smith *et al.* (3)).

Friedman (4) has recently described a somewhat complicated method for serum, in which, after proteins are precipitated by trichloroacetic acid, bromide is oxidized to bromate by hypochlorite, excess of hypochlorite is removed, and the bromate is estimated iodometrically.

A few years ago Wikoff *et al.* (15) described a method which consisted in the liberation of bromine by persulphuric acid, in the Folin-Wu filtrate, development of a violet colour (due to bromo-fuchsin) by addition of a fuchsin solution, and removal of interfering colour with fuller's earth. The authors stated that comparison with incineration methods indicated that all bromine is present in the Folin-Wu filtrate. Their figures for 10 normal human bloods varied from 0.6 to 1.6 and averaged 0.88 mgm. per 100 cc. They inferred that the method could be used for large amounts of bromine but quoted no data to substantiate this. In our hands this method showed a maximum colour development at about ten times normal values. Hence to employ it for clinical purposes great dilution would be necessary, with consequent potentially high error.

We have devised a simple method, also using persulphuric acid, and adapting Ulrich's procedure, as described by Mattice (6, p. 175), but without the initial oxidation of organic matter.

A mixture of sulphuric and persulphuric acids liberates the respective halogens from chloride, bromide, and iodide. Except in cases in which iodide has been administered, the iodine content of blood can be ignored, so that it is only necessary to determine the conditions in which bromine will be completely liberated from bromide, but chloride will not be appreciably decomposed.

Method

Link in series three 125 cc. gas-washing bottles (high form), exposing as little of the connecting rubber tubing as possible. Connect the third bottle to a suction pump.

Introduce into each of the second and third bottles 50 cc. of 2.5% potassium iodide, freshly made up.

Pour into the first bottle, in the order stated, 20 cc. of Folin-Wu, or, preferably, zinc hydroxide filtrate from blood (representing 2 cc. of blood, and prepared as stated below), 15 cc. of water, and 5 cc. of persulphuric acid in sulphuric acid. The persulphuric acid solution is prepared by adding 0.2 gm. of potassium persulphate (weighed with rough accuracy) to 5.0 cc. of 50% sulphuric acid (by volume) and shaking up. Most of the persulphate dissolves, and the solution is transferred to the washing-bottle after three minutes.

Immediately close the bottle and start suction. Aspirate rapidly for 30 min.

Disconnect, add the two iodide solutions together, with adequate washing, and titrate the liberated iodine against 0.01 *N* thiosulphate, using starch as indicator.

Preparation of the Folin-Wu filtrate: To one volume of oxalated blood add seven volumes of distilled water and shake gently to luke the blood. Then add one volume of 10% sodium tungstate and mix, and finally add very slowly, with constant shaking, one volume of two-thirds normal sulphuric acid. After five minutes filter off the chocolate-brown coloured precipitate. (Cf. Folin and Wu (2)).

Preparation of the zinc hydroxide filtrate: To one volume of oxalated blood add seven volumes of distilled water and shake up gently. Then add one volume of 10% zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), and one volume of accurately prepared 0.5 *N* sodium hydroxide. Shake up and filter. (Cf. Somogyi (10, 11).)

Calculation. No. of cc. of thiosulphate $\times 0.8 \times 50$ gives no. of mgm. of bromine per 100 cc. of blood.

Accuracy. Results are very slightly too low. Thus in typical examples the value 46.5 mgm. per 100 cc. was obtained instead of the true value 50 mgm., 244.5 mgm. instead of the true value 250 mgm.

Experimental Part

In carrying out the experiments on which the method is based, the volume of fluid in Bottle 1 was invariably adjusted to 40 cc. by varying the amount of water added. Initial experiments showed that separate addition of persulphate and sulphuric acid gave results that were much too low. Subsequently, therefore, the persulphate was always dissolved in the acid before addition, and the order detailed above was invariably used. In all cases 0.2 mgm. of persulphate was used.

The error of titration was almost certainly always less than one drop of thiosulphate, corresponding to between 0.02 and 0.03 mgm. of bromine.

Effect of Varying the Amount of 50% Sulphuric Acid Used

Pure bromide solution was employed, without addition of blood filtrate. The results are shown in Table I

The results suggest that with amounts of bromine varying from 1 to 15 mgm. 5 cc. of 50% sulphuric acid is adequate. It will be noticed that almost all the results are slightly low.

Measurement of the Upper Limit of Acidity Possible Without Appreciable Decomposition of Chloride

To one of a pair of solutions 1 cc. of 1% sodium chloride solution was added (displacing 1 cc. of water), thus introducing 6 mgm. of chloride-chlorine, approximately that present in 20 cc. of a blood filtrate. Parallel determina-

TABLE I

EFFECT OF VARYING THE AMOUNT OF SULPHURIC ACID

Volume of acid, cc.	Bromide-bromine taken, mgm.	Bromine found, mgm.	Average, mgm.
0.5	1.00	0.34	(0.34)
1.0		0.64	(0.64)
2.0		0.93	(0.93)
4.0		0.98	(0.98)
5.0		0.99, 0.86, 1.08	1.01
10.0		0.91, 0.93	0.92
1.0	2.00	1.57, 1.96	1.76
2.0		1.83, 1.96	1.89
5.0		1.85, 1.97, 2.00, 1.87	1.92
6.0		1.97	(1.97)
8.0		2.01, 1.95	1.98
10.0		1.89, 1.98, 1.93	1.93
15.0		1.94	(1.94)
5.0	0.20	0.17	(0.17)
		0.40	(0.35)
		3.00	(2.89)
		4.00	(3.90)
		5.00	4.91
		10.00	9.70
	15.00	9.84, 9.64, 9.56, 9.81, 9.55, 9.76, 9.76, 9.87 14.86	(14.86)
10.00	3.00	2.89	(2.89)
	4.00	3.90	(3.90)
	5.00	4.99	(4.99)
	10.00	9.83	(9.83)

tions were carried out, and the results (calculated as bromine) are shown in Table II.

Above 7 cc. of acid the results seem uncertain. Evidently 5 cc. of acid is sufficient for 15 mgm. of bromine and liberates no significant amount of chlorine.

Tests with Blood Filtrate and Added Known Amounts of Bromide

Mixed blood samples from patients who had received no bromide medication were used in preparing Folin-Wu and zinc hydroxide filtrates. Samples of such filtrates were analysed without bromide addition and usually gave perfectly negative tests for bromide. (When a trace of bromide was found, enquiry showed that the mixed sample contained blood from a patient who was receiving bromide medication; the sample was rejected.) The method is insufficiently delicate to detect the normal amount of bromine in blood.

Known amounts of bromide solutions were added to 20 cc. of Folin-Wu or zinc hydroxide filtrates in the first bottle, water was added to 35 cc., and then 5 cc. of the acid mixture. The results are shown in Tables III and IV. (The individual results for pure bromide solutions are given in Table I.)

TABLE II
EFFECT OF VARYING ACIDITY UPON DECOMPOSITION OF CHLORIDE

Volume of 50% sulphuric acid, cc.	Bromide-bromine taken, mgm.	Bromide-bromine found, mgm.	
		No chloride added	Chloride added
1.0	2.00	1.96	1.96
		1.57	1.69
2.0		1.83	1.72
		1.96	1.96
5.0		1.85	1.94
		2.00	2.05
		1.97	1.97
6.0		1.94	1.98
7.0		1.97	2.02
8.0		2.01	2.04
		1.95	2.04
9.0		1.90	1.95
10.0		1.98	2.36
		1.93	2.03
11.0		1.90	1.96
12.0		1.89	2.43
15.0		1.94	2.63
5.0	5.00	4.84	4.79
	10.00	9.55	9.74
		9.76	9.74
		9.76	9.76
	15.00	14.86	14.74

TABLE III
ESTIMATION OF BROMINE IN BLOOD FILTRATES CONTAINING KNOWN AMOUNTS

Bromide-bromine taken, mgm.	Amount of bromine found, mgm.				
	Pure bromide solution	Bromide solution plus Folin-Wu filtrate		Bromide solution plus zinc hydroxide filtrate	
	(Average)	Actual values	Mean	Actual values	Mean
0.20	0.17	0.03, 0.04, 0.00, 0.00	0.02	0.14, 0.16	0.15
0.50	—	0.42	(0.42)	0.43, 0.47	0.45
1.00	1.01	0.81, 0.79	0.80	0.95, 0.92	0.93
2.00	1.92	1.98, 2.03, 1.76	1.92	1.92, 1.97	1.95
3.00	2.89	2.71, 2.70	2.70	2.87, 2.88	2.87
5.00	4.91	4.70, 4.97, 4.74	4.80	4.89, 4.89	4.89
10.00	9.70	9.70, 9.88, 9.74, 9.78	9.78	9.81, 9.65, 9.88	9.78

Tables III and IV indicate that with zinc hydroxide filtrates the degree of error is negligible for clinical purposes; the figures for the Folin-Wu filtrates are less satisfactory, especially when the amount of bromine is small.

It may be noted that a single experiment with dibromtyrosine showed that under the conditions laid down it was not decomposed.

nitric acid and silver nitrate added. No precipitate of silver iodide was formed, indicating absence of any iodide in the residual fluid.

The results suggest that when all iodide has been decomposed, but before all the iodine has been aspirated off, in presence of the strongly oxidizing acid mixture a variable amount is oxidized to iodate.

They indicate also the strong possibility of conversion of some bromine to bromate, though, as Tables I to III indicate, to a much smaller extent. However, the larger the amount of free bromine the greater the amount of bromate that should be formed, and actually the loss is greater, the greater the amount taken.

It would thus appear that the most probable causes for the slight loss of bromine found to occur are, under the conditions of the estimation, formation of a minute amount of bromate, and, especially if the Folin-Wu blood filtrate is used, bromination of certain compounds in blood such as glutathione and ergothioneine.

Some Results of Employment of the Method

The following are typical:

(1) Mrs. H. A., weight 140½ lb. Given triple bromides containing 1.48 gm. of bromine daily. Blood-bromine was estimated on the Folin-Wu filtrates. Results are shown in Table VII.

TABLE VII
BLOOD-BROMINE. MRS. H. A.

Time, days	Bromine found, mgm. per 100 cc.
0	0.00
4	16.0
7	27.2
11	28.7
14	39.0
18	41.0
22	54.5
25	49.0

(2) Mrs. J., weight 135 lb., after 24 days on triple bromides containing 3.94 gm. of bromine daily, gave, using the Folin-Wu filtrate, the value for blood bromide-bromine of 146 mgm. per 100 cc.

(3) Mrs. C., weight 125 lb. Given triple bromide containing 1.48 gm. of bromine daily for 14 days. Blood bromine was estimated on the zinc hydroxide filtrate. Results are shown in Table VIII.

(4) Miss H., weight 124 lb. Given triple bromide containing 2.96 gm. of bromine daily for 14 days. Blood bromide was estimated on the zinc hydroxide filtrate. Results are shown in Table VIII.

TABLE VIII
BLOOD-BROMINE. CASES 3 AND 4

Time, days	Blood bromide-bromine, mgm. per 100 cc.	
	Mrs. C	Miss H
0	0.0	0.0
3	11.0	22.8
7	21.4	54.1
10	32.1	77.6
14	46.6	107.6
17	30.0	93.4
21	25.2	72.8

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A STUDY OF THE EFFECT OF CERTAIN DIETARY FACTORS ON THE PRODUCTION OF TAR-CARCINOMA IN MICE¹

BY A. T. CAMERON², SARA MELTZER³, AND J. M. LEDERMAN⁴

Abstract

A review of the literature suggests that a relationship exists between diet and the experimental production of skin carcinoma in mice, as far as the time of first appearance of tumours is concerned. The dietary factor or factors of importance in this respect have not yet been ascertained, although the recent work of Baumann and his collaborators suggests that a diet rich in fat accelerates the appearance of skin carcinoma.

There is no good evidence in the literature that any vitamin acts as an inhibitory factor to experimentally produced cancer of any kind with the sole exception of hepatomata produced by butter-yellow and inhibited by riboflavin.

Conclusions concerning diet and skin carcinoma in mice cannot be applied to any other form of cancer in mice and other animals, including clinical cancer in man.

The experiments reported in this paper suggest that the carcinogenic agent employed is of much greater importance than any dietary factor or any pretreatment of mice or their progenitors in determining the incidence of skin carcinoma. With a sufficiently powerful agent the incidence is 100% in an ordinary mouse colony, and the appearance of the cancer is rapid. With a weak agent such as dibenzanthracene the incidence is low, and the appearance of the cancer may be markedly delayed.

With the dietary proteins tested, in the amounts employed, no evidence was obtained that protein is a dietary factor affecting the time of onset of skin carcinoma.

No evidence was obtained that the content of essential unsaturated fatty acids is a dietary factor affecting the time of appearance of skin carcinoma, but too few animals were employed to permit final judgment on this point.

A slight modification of the Mackenzie-McCollum diet for rats (essentially cane sugar and casein, with the necessary vitamins, minerals, etc., added) proved only moderately successful for mice. Their life span was shortened, and maintenance of body weight was less perfect.

A peculiar syndrome is described, which developed in mice on this modified Mackenzie-McCollum diet containing an excess of the essential unsaturated fatty acids, and it is suggested that this excess perhaps can cause relative deficiency of some vitamin.

The Waddell-Steenbock diet for rats proved a suitable vehicle for testing protein modifications of the diet. However, the modified diet, while permitting mice to live to a considerable age, did not appear to be satisfactory for reproduction. The maximum age span in the experiments was 895 days.

The long survivals, with dibenzanthracene as tarring agent, were not obtained with either gas-works tar or benzpyrene.

The slow onset of a cancer, with dibenzanthracene as tarring agent, makes it unsuitable for such experiments. The most delayed appearance of a tumour, with this carcinogen, was 104 wk. from commencement of tarring.

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Introduction

Voegtlin (35) has pointed out that it is logical to consider that diet may be a possible modifying factor in carcinogenesis, since tissue cells are highly organized chemical units, whose normal functions depend upon a continuous supply of chemical compounds essential for production of energy and their own specific compounds and the regulation of their own activity.

Much work, by many investigators, involving great effort and much time, has been performed in attempting to discover the effect of diet on the experimental production of cancer. So far the results are suggestive but confusing.

It is frequently difficult to change the amount of one constituent of a diet without affecting the proportions of all the constituents. It is doubtful if this difficulty has been sufficiently considered in deducing many of the conclusions to be found in the literature.

The results of the present study are largely negative, as far as its initial purpose is concerned. Certain incidental observations seem worth recording. It has also seemed useful to review the literature rather thoroughly.

It has been clearly demonstrated by Baumann, Jacobi, and Rusch (2) that results concerning diet and cancerous growths produced by one specific method cannot be applied to cancerous growths produced by some other method without experimental verification. It follows even more forcibly that they cannot automatically be applied to clinical cancer. Hence, in considering the literature, each type of cancer should be considered separately.

Review of Previous Literature

Experimental Skin Carcinoma Produced by 'Tarring' or Painting with Other Carcinogenic Agents

Development of such carcinoma is promoted by a diet rich in fresh liver (24) or to which has been added an aqueous extract of liver, pancreas, or intestinal mucosa (25). The antianaemic factor of liver is not responsible for this effect (38). Such development (in the rabbit) is said to be inhibited by a diet rich in brain, or albumin, while addition to the diet of mice of dry pig's brain, or ether extracts of brain, thymus, or bone marrow, is said to be inhibitory (25, 26).

A diet rich in olein and protein and poor in carbohydrate is said to inhibit, while one rich in carbohydrate and palmitin and poor in protein is said to favour, development of carcinoma (16). Such a claim is difficult to analyse.

A diet high in fat accelerates production of tar-carcinoma in rabbits (27). One rich in butter increases the incidence in tarred mice (39). There seems no doubt that a diet rich in fat does accelerate the production of skin carcinoma, whether produced by tar or by methylcholanthrene (2, 20, 21), the tumour-promoting activity seeming to reside in the fatty acid radical (21), though there is some suggestion that the effect may really be partly due to an accompanying relatively high calorie intake (22).

The response of mice to skin painting with methylcholanthrene is said to be influenced by the amount of cystine provided by the diet (40) though it is doubtful if this affects the carcinogenic response. When *p*-thiocresol (which contains the sulphydryl group believed to stimulate cell proliferation) is applied to the skin of mice along with dibenzanthracene, resulting incidence of skin carcinoma is said to be reduced (28). (Claims have been made that arginine retards cell differentiation, while glutamic and aspartic acids and tyrosine help it, with the resulting suggestion that the immaturity of cancer cells may be associated with an imbalance between these amino acids (18). There is evidence that lysine is necessary for growth of both normal and malignant cells (36, 37).)

Burk and Winzler (5) have dealt exhaustively with the literature concerning possible relationships of vitamins to different forms of experimental cancer, and their considered view seems to be: "At the present time concrete evidence showing peculiar or specific relationships between vitamins and malignant growth is generally rather lacking." The only definite exceptions seem to be the effects of riboflavin and biotin on the production of hepatomata by butter-yellow (*vide infra*).

Davidson (9) fed groups of mice two very different diets selected as respectively rich or poor in vitamin E. The first diet contained much wheat germ and wheat germ oil. The animals were tarred with a potent gas-works tar. He considered that his results showed a more delayed and a lower incidence of production of carcinoma in mice on the diet rich in vitamin E, although (one of the present authors having acquainted him with the great general differences in the chemical composition of his two diets) he stressed also the richness of the "anti-carcinogenic" diet in thiamin and riboflavin, etc. "so that the specific factor or combination of factors which confers greater resistance on the mice cannot yet be stated."

In subsequent papers (10) he used diets of a more controllable nature, one of which was considered to be richer in vitamins A, B, and E than the other, and slightly poorer in fat. He carried out a complicated series of experiments with these diets; the number of animals used in each single experiment is not large enough to justify definite conclusions. Nevertheless, definite conclusions were drawn: "The tumour threshold can be lowered by tar-irritation, low vitamin content diet, and breeding, thereby obtaining 100 per cent of cancer. When the offspring of susceptible mice are divided and placed on two different diets, one low in vitamin content and the other high, the mice on the low-vitamin content diet develop tar-carcinoma as usual, but few mice on the high-vitamin content diet develop the growth. When susceptible mice are placed on a high-vitamin content diet, untreated, and allowed to breed naturally for a few generations, then placed back on a low-vitamin content diet and kept under the same conditions as the susceptible mice to produce tar-carcinoma, few are found with the growth present."

His last two papers (11) deal chiefly with clinical applications of his conclusions from his own experiments, complicated by the injection of extracts of young mice or rats or chick embryos.

Haddow and Russell (17) checked Davidson's work, using two groups of mice on a constant ration, fortified for one group by a relatively large amount of wheat germ oil and wheat germ meal. Benzpyrene was used as tarring agent. No inhibitory influence and no significant delaying effect were produced by the wheat germ and its oil, and it was thought that such effects might have been masked by the much more highly potent carcinogenic agent used.

Severi (32) was also unable to confirm Davidson.

In 1937 Cameron and Meltzer (6) published the results of experiments that followed Davidson's earlier work (9) as closely as possible. The same two diets were employed. The results did not completely confirm Davidson's conclusions. They indicated that the incidence of carcinoma was approximately the same on the two diets, but that its development in mice on the diet richer in certain vitamins tended to be delayed. The difference in the results may have been partly due to difference in techniques. In the experiments of Cameron and Meltzer all animals were isolated in separate cages, and lived longer and in better condition than Davidson's mice, injuries from fighting, etc., being prevented.

The essential results are shown in Tables I and II, reproduced from their paper. The "bad diet" was vitamin-poor, the "good diet" vitamin-rich.

Results of a typical experiment are shown in Fig. 1, which indicates that the time of first appearance of a tumour is slightly but not markedly delayed on the "good diet", while the duration of life is not greatly affected.

TABLE I
RELATIVE CARCINOGENIC EFFECTS OF TWO DIETS

	Bad diet	Good diet
Animals developing carcinoma	38 (90%)	42 (86%)
Animals developing papilloma	2 (5%)	3 (6%)
Animals developing no growth	2 (5%)	4 (8%)
	42 (100%)	49 (100%)

TABLE II
INTERVAL BETWEEN COMMENCEMENT OF TARRING AND APPEARANCE OF FIRST GROWTHS (WK.)

Bad diet			Good diet		
No. of animals	Extreme periods	Average	No. of animals	Extreme periods	Average
40	7 - 54	14	47	8 - 81	20

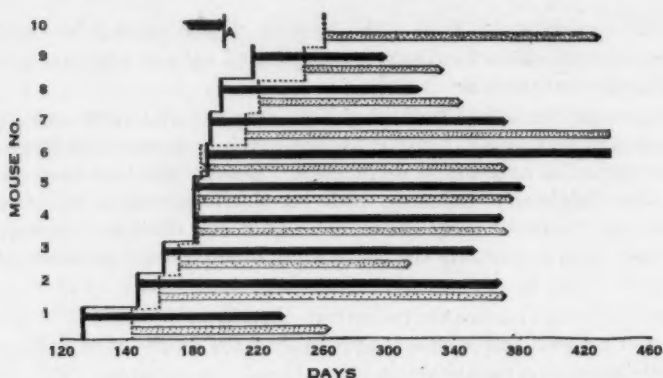


FIG. 1. Vertical lines indicate times at which tumours were first observed, reckoned from time of commencement of tarring. Each 5 mm. vertical line corresponds to one mouse. The horizontal arrows drawn from the vertical lines indicate time till death of the animal. Blunted arrows indicate that the animal was killed. Continuous lines and solid arrows are for mice on the "bad diet"; dotted lines and mottled arrows are for those on the "good diet". "A" indicates an animal showing no tumour, but only hyperplasia.

The two diets (Davidson's original diets) were very different. Approximately, they had the following composition:

	Bad diet, %		Good diet, %
Milk	12.0	Milk	25.9
Carrot	27.0	Lettuce	48.5
White bread	45.2	Wheat germ	22.4
Lard	8.6	Wheat germ oil	3.2
Oats	7.2		
	100.0		100.0

Referred to the ordinary diet tables they analysed to the following compositions:

	Bad diet, %	Good diet, %
Carbohydrate	58	40
Protein	13	31
Fat	27	24
Ash	2	5
Vitamin A	+	+
Thiamin	+	++++
Riboflavin	+	+++
Vitamin D	+	+
Vitamin E	+	+++++
Essential unsaturated fatty acids	(+)	+++++

Obviously the delaying effect of the one diet (or the accelerating effect of the other) on the induction of a carcinoma could be due to (i) differences in protein content, either qualitative or quantitative, (ii) differences in mineral content, either qualitative or quantitative, (iii) difference in content of thiamin, riboflavin, or vitamin E or some combination of them, (iv) difference in content of the essential unsaturated fatty acids (linoleic acid, etc.), or (v) differences involved in the balance of the two diets. It is to be noted that both are rich in fat.

Cameron and Meltzer (6), using modifications of the Waddell-Steenbock diet for rats, contrasted the effects of diets rich and poor in thiamin, riboflavin, and vitamin E, but could find no evidence that variations in dietary content of these three vitamins affected the time of appearance of skin carcinoma in tarred mice. It is possible that the number of animals tested was too few to permit definite conclusions on this point.

No clear cut data are available relating the minerals of diet to production of experimental cancer (33).

Notes on Other Types of Experimental Cancer

High fat diets accelerate the tumours produced in mice by ultra-violet light (2, 3), but do not affect the production of sarcomata following injection of methylcholanthrene (22), nor increase the incidence of spontaneous breast tumours in mice (22).

Claims have been made that rats fed ether-extracted unrefined wheat germ oil develop abdominal sarcomata (29, 14, 30, 31), but have not been confirmed (12, 7, 18, 1, 15, 13).

Hepatomata produced by feeding butter-yellow (*p*-dimethylaminoazobenzene) to rats are inhibited by casein and riboflavin; the inhibition is lessened by biotin (34).

The mother's milk of C3H mice promotes development of mammary cancer not only in its own offspring but also in foster-nurslings of a strain of low tumour incidence (4).

Conclusions from the Literature

Certain diets do affect the time of appearance of skin carcinoma in tarred mice. There is as yet no evidence to show whether this effect is due to a single or to multiple factors.

The experiments of Cameron and Meltzer with animals isolated in separate cages indicate that such animals tend to live longer and in better general health, so that in consequence some develop skin carcinoma at an age that is usually not reached by any of groups of animals kept together in a cage, the more customary procedure. This suggests that various conclusions in published data that certain experimental procedures *lessen* the incidence of skin carcinoma should perhaps be modified to the inference that such procedures *delay* the appearance of tumours, shorter-lived animals often dying before such tumour growth is obvious.

A diet high in fat is procarcinogenic, in the sense of hastening the appearance of a tumour produced by tarring or by ultra-violet light, but not by injections of carcinogenic agents. Nor does it increase the incidence of spontaneous mammary tumours in mice.

There is no good evidence that any vitamin acts as an inhibiting factor, except in the specific case of riboflavin, inhibiting production of hepatomata by butter-yellow.*

Conclusions concerning skin carcinoma in mice cannot be applied to any other form of cancer in mice or in other animals, including clinical cancer in man.

Aim of the Present Research

This is a continuation of the work of Cameron and Meltzer. The series of experiments now reported was commenced in the fall of 1939, and was in large part concluded in 1942. It was designed to test two dietary variations, (A) the effect of varying the protein content on the rate of development of skin carcinoma in mice painted with a carcinogenic agent, and (B) the effect of varying the content of essential unsaturated fatty acids in the diet on such rate of development.

At the time of commencement of the work Baumann's results on fat content of the diet were still unpublished.

Experimental Part

(A) EFFECT OF VARYING THE PROTEIN CONTENT OF THE DIET

Cameron and Meltzer (6) showed that the Waddell-Steenbock diet for rats could be successfully used for mice. It was accordingly modified suitably to contain different proportions of proteins. The following three rations were used:

	Diet 1, gm.	Diet 2, gm.	Diet 3, gm.
Yellow corn	82	102	0
Crude casein	20	0	4
Wheat flour	0	0	145.33
Linseed meal	20	20	20
Alfalfa meal	2.67	2.67	2.67
Bone ash	1.33	1.33	1.33
Sodium chloride	0.67	0.67	0.67
Klim	63.33	53.33	0
Vitamin B complex	0.5	0.5	0.5
Butter	10.0	20.0	10.0
Olive oil	0	0	16
	200.5	200.5	200.5

* The following very recent note seems important enough to merit reference, although treatment was by injection and not orally. R. Leuchtenberger, C. Leuchtenberger, D. Laszlo, and R. Lewisohn report in *Science* of Jan. 12, 1945 (101: 46) that injections of the vitamin "folic acid" in amounts of the order of 5 μ m. daily for 4 to 6 wk. caused complete disappearance of 38 out of 89 spontaneous breast cancers in three different strains of mice, while only one new tumour appeared during several months. In 60 controls no tumours disappeared and 14 new ones appeared.

In making up these diets all the materials, except the butter (and olive oil) were mixed together thoroughly, and then this was mixed in to form a paste. Parke-Davis 'Kapseals Kombex' were used for the B complex. These varied slightly in content during the course of the research, those available in the latter stages containing per 0.5 gm. 1.5 mgm. thiamin chloride, 0.5 mgm. riboflavin, 0.25 mgm. pyridoxin hydrochloride, 0.3 mgm. pantothenic acid, 5 mgm. nicotinic acid, and unstated amounts of other vitamins of the complex from a liver concentrate.

Analysed by the ordinary dietary tables the three rations had the following percentage contents of proteins and fats:

	Diet 1		Diet 2		Diet 3	
	Protein, %	Fat, %	Protein, %	Fat, %	Protein, %	Fat, %
Yellow corn	3.8	0.8	4.7	1.0	—	—
Wheat flour	—	—	—	—	7.9	0.8
Casein	10.0	0.0	—	—	2.0	0.0
Linseed meal	3.2	4.6	3.2	4.6	3.2	4.6
Alfalfa meal	0.4	Trace	0.4	Trace	0.4	Trace
Klim	8.7	8.9	7.1	7.5	—	—
Butter	0.0	4.1	0.0	8.2	0.0	4.1
Olive oil	—	—	—	—	—	8.0
	26.1	18.4	15.4	21.3	13.5	17.5

Dr. F. D. White was good enough to carry out analyses for water, protein (calculated from total nitrogen), and fat on several samples at different times, using the customary procedures. He obtained the following results:

	Diet 1			Diet 2			Diet 3		
	Protein, %	Fat, %	Water, %	Protein, %	Fat, %	Water, %	Protein, %	Fat, %	Water, %
Sample 1	23.3	20.4	6.6	14.4	23.7	7.7	14.6	12.7	6.7
Sample 2	23.1	19.1	7.2	14.5	22.0	7.6	15.0	12.8	4.9
Sample 3	23.0	20.8	7.3	15.4	23.9	7.8	14.9	15.3	6.0

It will be seen that the protein values are in moderate agreement with the calculated values, that the fat values for Diets 1 and 2 are of the order calculated, but that for Diet 3 is definitely lower than the calculated figure.

Diet 1 can be considered as a high protein diet of good grade, Diet 2 a low protein diet of similar grade, and Diet 3 a low protein diet in which the protein is largely provided by wheat flour. In the first two the fat content is high (20% or more) and in the third definitely lower (15% or less).

The stock of mice used throughout the experiments was cross-bred from the various stocks used in the Cameron-Meltzer experiments. Many intervening generations had been fed ordinary laboratory diet.

Experiment 1

Three groups of mice were respectively placed on the three diets. Each mouse was fed 3 gm. daily until the end of the tarring period, and then 3.5 gm. daily. In addition each mouse was given from a dropper one drop of a standardized cod liver oil twice a week. Water was given ad lib. in a separate vessel. All uneaten food was removed each morning.

On this diet, which was almost all eaten, the mice grew normally; nearly all remained in good health, unless affected by the experimental treatment.

When the experiment was commenced no benzpyrene was available. It was decided to use 1,2,5,6-dibenzanthracene, although this is recognized as less carcinogenic and less rapid in action than either a potent gas-works tar or 3,4-benzpyrene. Animals were painted in the usual fashion twice weekly, on a small patch of skin of the back, just behind the head. Sodium sulphide solution was used as a depilatory agent, and its use repeated when necessary. The painting consisted in rubbing in a drop of a saturated solution of the dibenzanthracene in benzene.

An initial attempt to compare mice of the same sex and litter failed through occasional deaths from tarring and other extraneous causes, so that the total number of animals on each diet at the end of the tarring period had to be compared. The animals were born between Nov. 26 and Dec. 21, 1939, inclusive; the tarring period lasted from Feb. 20 to June 21, 1940. Six animals died during the tarring period or within a few days of its cessation; their deaths were considered as due to the toxic action of sodium sulphide or of dibenzanthracene. None of the six showed any trace of tumour growth. They were not included in the analysis of results.

All animals that developed growths, and almost all the others that showed no visible growth, were preserved in 10% formaldehyde, and subsequently examined grossly and histologically. Carcinomata were graded in accordance with the usual procedure for human carcinomata (cf. 6). The results are shown in Table III, in which all times mentioned are reckoned from the first day of tarring.

The results are somewhat confusing. The average life span of those that developed cancer is in each group somewhat longer than the life span of those that did not develop cancer. This is probably due to relatively early deaths in animals that would have developed cancer had they survived longer.

The average time of development of tumours was definitely earlier in animals on Diet 2 than in those on Diet 1, or Diet 3. Thus, one year (365 days) from the time at which tarring was begun, seven animals on Diet 2 showed growths, two on Diet 3, and one on Diet 1.

An attempt has been made to show this in Fig. 2, which shows the proportion on each diet that had developed cancer at any given time. To construct this figure each animal was given a percentage value in terms of the whole number of its group. Thus one animal equals 5.55% for Diet 1, 4.54 for Diet 2, and 6.25 for Diet 3.

TABLE III

RELATION OF DIETARY PROTEIN TO PRODUCTION OF SKIN CARCINOMA BY DIBENZANTHRACENE
(Experiment 1)

	Diet 1	Diet 2	Diet 3
Total number of animals	18	22	16
Number showing no tumour	11	12	7
of these hyperkeratosis was present in	2	3	1
Number showing growths	7 (39%)	10 (45%)	9 (56%)
Gradation of tumours: Grade IV	1	1	1
Grade III	0	2	0
Grade II	0	2	5
Grade I	5	5	3
Fibrosarcoma	1	0	0
Duration of life (days) of those showing no growths			
Extremes	186 - 590	174 - 817	394 - 734
Average	430	497	559
Duration of life (days) of those showing tumours			
Extremes	334 - 648	415 - 766	439 - 764
Average	550	553	571
Time (days) when first growth seen			
Extremes	184 - 648	163 - 682	234 - 732
Average	405	348	470

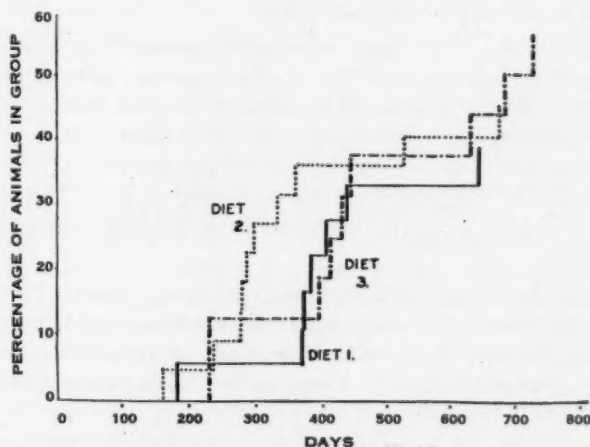


FIG. 2. Vertical lines indicate time of first appearance of tumours, reckoned from time of commencement of "tarring". Each vertical unit represents one animal, the length of the unit being proportional to the percentage value of the animal in the group.

The experiment fully confirms the lesser carcinogenic action of dibenzanthracene (as compared with tar) and the markedly delayed onset of carcinoma that can occur with its use. Thus on Diet 3 a skin carcinoma, Grade I, developed, which was not noticeable until the 732nd day (over 104 wk.) after

tarring commenced. The most delayed case reported by Cameron and Meltzer (6) using gas-works tar was observed after 81 wk.

It is also to be noted that Diets 2 and 3 both seemed more favourable to long life of the mice than Diet 1, and that isolation of the mice in separate cages permitted some of them to live to relatively great ages. Thus on Diet 2 one mouse lived 817 days from start of tarring, and actually 895 days in all; a second lived 791 days from start of tarring (860 days in all). On Diet 3 one mouse lived 764 days from start of tarring (840 days in all). The first two developed no growth, the third, as mentioned above, showed a Grade I carcinoma on the 732nd day.

Experiment 2

As the age figures of Experiment 1 show, the time that elapsed between its start and finish was nearly $2\frac{1}{2}$ years. It was impossible to wait for its near completion before beginning further experiments. Since its earlier results suggested that tumours developed more quickly on Diet 2 than on Diet 1, the second experiment was devised to test this further.

Thirteen pairs of male mice and 11 pairs of females, born between Jan. 4 and 12, 1941, were isolated in separate cages, and each group of 24 placed on Diets 1 and 2 respectively on Feb. 10, and then tarred with a potent gas-works tar (the same sample that had been used by Cameron and Meltzer) twice a week from Feb. 21 to June 27, 1941.

On the night of May 22-23 there was a sudden marked drop in temperature, to which apparently the mice on Diet 1 were somewhat more exposed in the animal room, so that 16 of them died within 24 hr. and another three days later. (The pathologist's report stated that one of these animals had a small papilloma at the site of tarring, the others hyperkeratosis only.) The remaining seven animals of this group survived the tarring period. Three on Diet 2 died during the tarring period, probably from sulphide poisoning. None showed growths.

The surviving groups of 7 and 21 animals could not properly be compared. All animals developed skin carcinoma. All surviving animals were killed between Sept. 30 and Oct. 5, 1941. The results of the experiment, such as they are, are given in Table IV. Times are estimated from the beginning of tarring.

On account of the marked disparity of numbers of the two groups stress can be laid neither on the much earlier appearance of a tumour in some mice on Diet 2, nor on the much greater incidence of Grade II carcinomata in this group.

The experiment does illustrate the remarkable difference in carcinogenic potency of the gas-works tar and dibenzanthracene, as is well shown in Table V. It is to be remembered that the mice used in both experiments were of the same mixed stock, bred on the same mixed diet.

TABLE IV
RELATION OF DIETARY PROTEIN TO PRODUCTION OF SKIN CARCINOMA BY TARRING
(Experiment 2)

	Diet 1	Diet 2
Number of mice	7	21
Number showing tumours	7	21
Carcinoma, Grade II	2	19
Grade I	5	2
Times (days) when tumours first observed		
Extremes	104 - 125	69 - 144
Averages	117	115

TABLE V
COMPARISON OF CARCINOGENIC POTENCY OF GAS-WORKS TAR AND DIBENZANTHRACENE

	Diet 1		Diet 2	
	Tar	Dibenzanthracene	Tar	Dibenzanthracene
Time of first appearance of a growth (days)				
Minimum	104	184	69	163
Maximum	125	648	144	682
Average	117	405	115	348

Experiment 3

Eighteen triads, each of the same sex and litter, forming three groups each containing 11 males and 7 females, were isolated in separate cages on July 24, 1941, and the groups fed respectively the three diets, 1, 2, and 3. One animal on Diet 2 died prior to start of tarring. Of the others seven in each group (including both males and females) were tarred with the gas-works tar from Sept. 23 to Dec. 23, and the remaining animals were painted twice a week during the same period with a 20% solution of 3,4-benzpyrene in benzene. The period of tarring was shortened with the idea that the onset of cancerous growths might be spread over a longer period than in Expt. 2, and thus enable more definite conclusions to be drawn. None of the animals died during the tarring period. The results of the experiment are shown in Tables VI and VII. Animals surviving 238 days from the start of tarring were killed and autopsied.

There does not seem to be any significant difference in the results for the three groups of animals and they therefore lend no support to the conclusions suggested by those of the first experiment.

There is obviously a marked difference between the effects of benzpyrene and gas-works tar. Animals treated with benzpyrene died earlier, developed tumours earlier, and developed malignant growths in 100% of cases. The differences are shown graphically in Fig. 3.

TABLE VI
RELATION OF DIETARY PROTEIN TO PRODUCTION OF SKIN CARCINOMA BY TARRING
(Experiment 3)

	Diet 1	Diet 2	Diet 3
Number of animals	7	7	7
Number developing a tumour	6	7	6
Grade IV	1	0	1
Grade III	0	4	0
Grade II	0	1	2
Grade I	4	2	2
Papilloma	1	0	1
Metastases	1	2	0
Number showing no tumour	1	0	1
Number alive at 238th day	1	0	1
Time (days) when growth first observed			
Extremes	93 - 198	93 - 128	93 - 156
Average	120	108	120

TABLE VII
RELATION OF DIETARY PROTEIN TO PRODUCTION OF SKIN CARCINOMA BY TARRING
(Experiment 3)

	Diet 1	Diet 2	Diet 3
Number of animals	11	10	11
Number developing a tumour	11	10	11
Grade IV	0	0	0
Grade III	2	3	1
Grade II	4	2	3
Grade I	5	5	7
Metastases	6	2	4
Number alive at 238th day	3	1	0
Time (days) when growth first observed			
Extremes	65 - 100	65 - 100	78 - 93
Average	86	84	84
Average time of death of those that died	208	200	181

Conclusions from the Three Experiments

With the proteins tested, the quality and amount do not appear to affect the time of onset of a skin carcinoma in the mouse by application of a carcinogenic agent.

Experiment 4

In the earlier stages of the preceding experiments, when there seemed some degree of evidence that quantity or quality of dietary protein might be a factor in determining the time of appearance of skin carcinoma, an experiment was begun, on the assumption that such factor did exist, to see whether mice

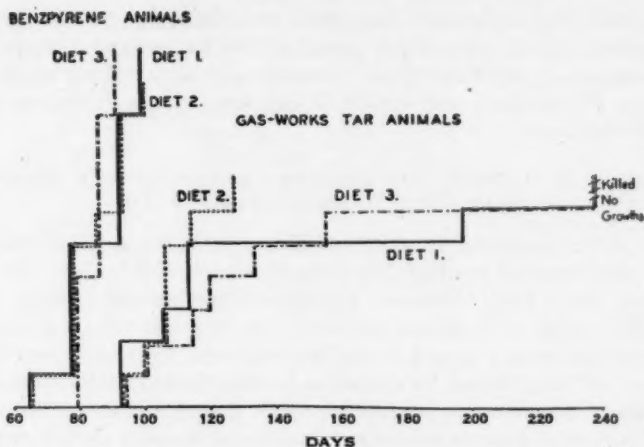


FIG. 3. Vertical lines indicate time of first appearance of tumours, reckoned from commencement of tarring. Each vertical unit of 5 mm. represents one animal. An animal on Diet 1 and another on Diet 3, killed on the 238th day, exhibited no tumours.

bred on one or other of the three diets might show increased or decreased susceptibility to tarring after several generations, when compared with mice similarly bred on the other diets. The final conclusions from the three experiments indicate that such breeding experiments would be futile, as far as these particular diets are concerned, but, in any case, all three diets seemed to be imperfect as far as reproduction is concerned.

Closely related animals, raised on stock diet, were mated in the summer of 1941, and the resulting litters were used in this experiment, three groups of pairs being segregated and kept on Diets 1, 2, and 3 respectively. Their young were maintained on the respective diets after weaning, and in this way attempts were made to obtain successive generations of what may be termed 'Diet 1 animals', 'Diet 2 animals', and 'Diet 3 animals'. It must be remembered that the first generation in each case was partially reared on stock diet (bread, milk, and scraps).

Diet 1 Animals.—Two second generation females did not become pregnant during a period at the end of which they were $7\frac{1}{2}$ months old. They were then transferred to the stock diet, and promptly dropped litters in 17 and 19 days respectively.

Diet 2 Animals.—Second and third generations were obtained, though the litters tended to be small and were frequently killed by the mothers or died young. Rearing of the litters appeared to be the chief difficulty.

Diet 3 Animals.—A number of second generation litters were obtained, but almost all consisted of only one or two animals, or were killed by the mothers. However, it was finally found possible to rear two litters with partial success, four surviving to adult age out of six animals in each litter.

Conclusion.—Although these diets seem so satisfactory for the continued and long life of mice, some factor is inadequate for successful reproduction or rearing of young, or both. Diet 2 seems most nearly adequate. (Certain recent work by Cerecedo and Vinson (8) suggests that such rations may be deficient in folic acid.)

(B) EFFECT OF VARYING THE DIETARY CONTENT OF THE ESSENTIAL UNSATURATED FATTY ACIDS (E.U.F.A.)

At first it was intended to select modifications of the Waddell-Steenbock diet that would permit marked differences in content of E.U.F.A. Dr. H. N. Brocklesby, then Chief Chemist, Fisheries Experimental Station, Prince Rupert, B.C., and a recognized authority on fats and oils, was consulted and advised against this procedure, as he considered that the content of these acids is not well established for numerous foodstuffs, and probably fluctuates considerably. He offered to provide a partially dehydrogenated oil containing no E.U.F.A. and also pure specimens of linoleic and linolenic acids (or mixtures of the two), and suggested that all other ingredients of the diet be extracted with ether, and then known amounts of hydrogenated oil and E.U.F.A. be incorporated, so that the amount of the latter in the diets would be controlled. This offer was accepted.

Experiment 5

This experiment became an attempt to solve the problem of finding a diet that would fulfil the following conditions: (i) it must be easily prepared in bulk; (ii) it must be suitable for incorporation of known amounts of E.U.F.A.; (iii) it must be adequate for normal growth and maintenance. (It is doubtful if this problem has been completely solved.) The experiment was complicated through the long distance (1500 miles) between the source of the hydrogenated oil and E.U.F.A. (Prince Rupert) and the place of experiment (Winnipeg), so that occasional shortages occurred and unsatisfactory substitutes had to be employed for short intervals.

The mortality in this experiment due to dietary defects was too great to permit any definite conclusions as to the effect of tarring.

Twelve sets of mice were isolated in individual cages early in February, 1940. Each set consisted of three animals of the same sex and litter. They were placed on three diets, alike except that one (A) was considered to contain a normal amount of E.U.F.A., the second (B) an amount only just sufficient to prevent occurrence of the fat deficiency disease of Burr, and the third (C) a definite excess of E.U.F.A.

Diet A contained 0.15% E.U.F.A., B 0.01%, and C 0.50%. A mixture of linoleic and linolenic acids was used, in which two-thirds or more was always linoleic acid. Adequate amounts of vitamins A and D were at first incorporated by Dr. Brocklesby in the hydrogenated oil, but subsequently he furnished us with a tuna liver oil concentrate rich in these vitamins, and this was added during mixing.

At first the basic diet employed corresponded to the Waddell-Steenbock diet for rats, the constituents having been ether-extracted, and the amount of each ingredient corrected accordingly. The general composition was:

Ether-extracted yellow corn	42.9	parts
" crude casein	3.1	"
" linseed meal	5.4	"
" alfalfa meal	1.27	"
" Klim	25.0	"
Bone ash	0.67	"
Sodium chloride	0.33	"
Vitamin B complex (Kapseals)	0.20	"
Hydrogenated oil and E.U.F.A.	21.33	"
	100.2	

The E.U.F.A. were incorporated with suitable amounts of the oil, and then these were mixed with much larger amounts to ensure thorough distribution of the E.U.F.A. The other constituents were thoroughly mixed together, and then the oil plus E.U.F.A. mixed in to a paste.

The mice were given 3.0 gm. of the mixture daily, with unlimited water. The food was almost all eaten, uneaten residues being removed each morning.

Tarring with 1,2,5,6-dibenzanthracene (using sodium sulphide as depilator) was commenced on Feb. 19, performed twice a week, and continued to June 20.

It soon became obvious that thorough extraction of the materials with ether was not only costly and time-consuming, with lack of special apparatus for the purpose, but that even after many extractions all ether-soluble material was not removed, so that the content of E.U.F.A. was not definitely under control.

In 1939 Mackenzie, Mackenzie, and McCollum published a paper in which it was shown (23) that ether extraction is often very inadequate for removal of all lipides from material such as casein, alcohol extraction being more effective. Dr. Brocklesby examined the casein we were using and found that the total linoleic and linolenic acid content in it did not exceed 0.03% and probably was only about 0.004%. Nevertheless he advised that the material be extracted twice with two or three times its volume of methyl alcohol plus ethyl alcohol (in ratio 1 : 2).

Mackenzie, Mackenzie, and McCollum showed further that a diet consisting chiefly of sucrose and alcohol-ether-extracted casein, with suitable additions of cystine, minerals, vitamins, and E.U.F.A., was adequate for normal growth and reproduction of rats. We modified their diet as follows, extracting the casein as recommended by Brocklesby:

	%
Powdered cane sugar	65.0
Extracted casein	22.1
Cystine	0.055
Salt mixture	6.74
B complex (Kapseals)	0.555
Hydrogenated oil + E.U.F.A. + A + D	5.55

The tuna oil concentrate was estimated to contain 450,000 I.U. of vitamin D and 250,000 I.U. of vitamin A. Two drops were added to 600 cc. of the hydrogenated oil during mixing.

This diet was used from Mar. 15, 1940. Initially 3 gm. were fed each mouse daily. This proved inadequate to maintain weight and after a month was increased to 3.5 gm. daily. This was almost all consumed by all animals as long as they remained healthy.

During the tarring period several deaths occurred that were attributed to the toxicity of the sodium sulphide. Also, early in this period, for 2 or 3 wk., the diets by accident contained too small an amount of the B complex; some deaths, which may have been due to a vitamin deficiency, occurred subsequently in each group. In late August, 1940, a supply of hydrogenated oil was delayed, and in the emergency a corresponding volume of pure glycerol was used as mixing agent. The E.U.F.A. mixed in it to give a good emulsion, but the A and D concentrate did not appreciably dissolve. This emulsion had to be used for 3 wk.; no apparent ill effects resulted.

Some mice on diet C (excess E.U.F.A.) developed a peculiar syndrome. At least four animals were affected. The condition was characterized especially by loss of hair from the fore part of the body. The face and head were first affected, then the abdomen, and even the legs. The four animals all died during a period of seven weeks following cessation of tarring. In the most extreme case the mouse might almost be described as in a condition of complete skin denudation of the fore part of the body, which exhibited a glistening, wet appearance. No animals on Diet A showed this syndrome. One animal on Diet B showed some loss of facial hair only; the condition was not comparable.

Since the vitamin contents of the three diets were the same, the effect does not seem attributable to a vitamin deficiency per se. The essential feature of Diet C was excess of E.U.F.A., but such excess seems unlikely to produce such a marked effect. The possibility suggests itself that the effect may be attributable to the E.U.F.A. excess causing a relative deficiency of some one or other vitamin. This is the more likely since there was no definite example of this syndrome in the later experiment, in which there were fewer dietary complications.

None of the four animals exhibiting this syndrome showed any growths at death.

Owing to the various causes mentioned only 17 of the initial 36 animals survived the tarring period for any considerable time. Of four on Diet A the final survivor died on the 539th day from commencement of tarring. None of these four exhibited any tumour. Of eight on Diet B the final survivor died on the 467th day. It alone developed a tumour, a Grade I carcinoma, first seen on the 140th day. Of five animals on Diet C the last survivor died on the 237th day. It exhibited a papilloma, the others no tumours.

It is evident, from the protein experiments, that since dibenzanthracene was used as tarring agent, most of the mice died too early to exclude the

possibility of development of tumours had they lived a few months longer. Nor can any conclusions be drawn as to the effect of variations of E.U.F.A. in the diets.

Experiment 6

Eighteen triads of animals were isolated (as in Experiment 5) and feeding of the three diets commenced on June 10, 1940. There were no complications in this experiment, except for the short period when glycerol had to be substituted for hydrogenated oil. The animals were painted with dibenzanthracene twice a week from June 24 to Oct. 25.

As an indication that the diets were not perfect, it was found that, on the average, during the period June 10 to Nov. 18, 14 survivors on Diet *A* showed a loss of weight of 0.3 gm., nine on Diet *B* no loss or gain in weight, and 11 on Diet *C* a loss of 3.6 gm. Additional evidence seems to be provided by the unusually large proportion of deaths in all three groups during the tarring period or shortly thereafter. Thus, to the 40th day from end of tarring four of Group *A*, nine of Group *B*, and nine of Group *C* had died. None of these animals showed any signs of a skin tumour, but all were in poor condition. Only one animal on Diet *C* showed (very slight) symptoms of the peculiar syndrome that developed in Experiment 5. It is perhaps worth noting that the greatest survival was in the group fed a normal amount of E.U.F.A.

The results obtained with these survivors are shown in Table VIII.

TABLE VIII
RELATION OF ESSENTIAL UNSATURATED FATTY ACIDS OF DIET TO PRODUCTION OF SKIN CARCINOMA
BY DIBENZANTHRACENE
(Experiment 6)

	Diet <i>A</i>	Diet <i>B</i>	Diet <i>C</i>
Total number of animals	14	9	9
Number developing carcinoma	3	1	2
Grade IV	0	0	1
Grade I	3	1	1
Time (days) of first appearance of growth	256, 283, 329	310	262, 304
Period of survival (days)			
Extremes	317 - 417*	275 - 348	193 - 360
Average	376+	324	307

* Four animals (one with a carcinoma) survived to the 417th day and were then killed.

The difference in the proportion developing carcinomata in the three groups is insufficient, with the small number of animals in the groups, to permit any conclusion as to the effect of variations in the amount of E.U.F.A. in the diet.

The definitely earlier death rate of mice on Diets *B* and *C* is a further indication that Diet *A*, with an intermediate amount of E.U.F.A. is the most nearly adequate of the three.

While the fat content of the ABC diets (5.5%) is very much less than that in the protein experiments, the fewness of the animals and the very great differences in the two types of diet prevent the drawing of any conclusions as to the effect of quantitative variations in dietary fat on the production of skin carcinoma by tarring.

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